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Neuroendocrine Tumors and Chronic Inflammation: A Model for the Association Between Endocrine Feedback Mechanisms and Cancer Pathogenesis

Ana Paula Soares Santos

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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ANA PAULA SOARES SANTOS

NEUROENDOCRINE TUMORS AND CHRONIC INFLAMMATION: A MODEL FOR THE ASSOCIATION BETWEEN ENDOCRINE FEEDBACK MECHANISMS AND CANCER PATHOGENESIS

Tese de Candidatura ao grau de Doutor em Ciências Médicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Ideias

“As ideias!
As ideias não são tão simples
como deveriam ser.
São como linhas
que podem ser tortas ou direitas,
enviesadas ou torcidas
e depois estendem-se em novelos
densos e impossíveis de desfazer.
As ideias também podem ser tão complexas!
Como linhas sem desvios ou contornos,
como um fio de prumo esticado e inerte.
Uma coisa invisível porque sem desvio,
sem aviso de que vai sair da linha.
As ideias poderão ainda ser difusas ou impercetíveis,
conjunções caóticas ou injunções contraditórias.
Mas todas elas são poderosas num espírito de vontade livre,
vontade indomável, incondescendente e criadora.
Não somos nada sem ideias,
um ponto inicial do sonho,
As ideias!”

Carlos Pax (unpublished, 2019)

Será que eu sei

“Será que eu sei
o que é uma flor?
É um ramo
de pétalas de cor.

Será que eu sei
o que é o dia?
É o oposto da noite
com alegria…

Será que eu sei
o que é a música?
É um conjunto de notas
Que até me deixa “confúsica”!

Será que eu sei
o que é um livro?
Páginas escritas de histórias
Que até parece que as vivo.

Será que eu sei
o que é o amor?
É um sentimento
que nos dá calor.

Será que eu sei
o que é a vida, que para este poema
me inspira?“

Inês, 12 years (unpublished, 2018)

Dedicated to my beloved parents, who transmitted to me the value of work, study, knowledge and dignity.

Dedicated to my beloved daughter Inês, to whom I would like to transmit this values for life.

BIOGRAPHIC NOTE
The Role of the Wisdom of the Body in My Career

"…Como inversão da hierarquia das cadeias organizacionais, o cancro pode representar, também, a subversão biológica que pretende encontrar uma nova ordem, um novo equilíbrio. Pode representar o conflito de um corpo com a agressividade do meio e a consequente procura de um novo estádio ecológico. Ou seja, pode ser um sinal de evolução, um sinal de insatisfação do corpo com o conjunto de "atractores" que naquele momento o configuram. Pode representar aquilo que se designa por "transição de fase", quer dizer, a passagem de um comportamento periódico a um comportamento turbulento."

Paulo Cunha e Silva
O Lugar do Corpo – Elementos para uma Cartografia Fractal (Silva 1995)

Since I have memories, I remember I wanted to be a paediatrician just because I loved my paediatrics doctor, António Bártolo. I was lucky enough to succeed to go to medical school. During the medicine course at ICBAS, whose fundaments are based on the Abel Salazar`s principle that "a doctor that only knows about medicine neither medicine he knows", I was touched by endocrine physiology. I was fascinated by the mechanisms of endocrine feedback perfectly "created" in order to maintain homeostasis, in other words, the wisdom of the body (WOB) that always interacts with the environment changes towards a new equilibrium (Cannon 1939). Later, on the 3^{rth} year I was surprisingly overwhelmed by oncology at the pathology lessons taught by Professor Oliveira Torres about carcinogenesis (Pathology classes, third year medical school ICBAS). His talks describing cancer as an attempt of nature to survive through the formation of a new organ was thrilling. At that time, he was already addressing that "thyroid carcinoma emerged from a continuous stimulation of the gland by TSH, as in the case of iodine deficiency". These lessons made cancer beautiful to me, although it was an awful murderess disease. On the end of medicine course, clinical learning on endocrinology with Dr. Conceição Bacelar showed to be even more interesting than I suspected and when I had to choose my future speciality, I had no hesitation between paediatrics and endocrinology. One of my first oral presentations at the Endocrinology Department of Hospital Santo António, founded by Doutor Ignácio Salcedo, as endocrinology registrar was entitled

"Ectopic Hormone Syndromes" with the subtitle "Humours from Tumors". Hippocrates believed that the body had 4 *humors* (body fluids): blood, phlegm, yellow bile, and black bile. When the humors were balanced, a person was healthy. The belief was that too much or too little of any of the humors caused disease. An excess of black bile in various body sites was thought to cause cancer (Geldard 2000). Later on my training, me and Dr. Conceição Bacelar had the opportunity to treat two patients with endogenous hyperinsulinism and a VIPoma with octreotide, and these were among the first patients in Portugal, to whom that innovative therapy was prescribed in the early nineties.

In the end of my training in endocrinology, I had the opportunity to help Professor Helena Cardoso with her PhD thesis work about insulin resistance (IR) evaluation by the Minimal Model Euglycemic Clamp (Silva 1999). At that time, the work from Reaven (Reaven 1988), De Fronzo (DeFronzo, Tobin et al. 1979, DeFronzo, Ferrannini et al. 1983), Ferrannini (Ferrannini, Bjorkman et al. 1985) and others about the recent discovered mechanisms of IR, hyperinsulinemia and type 2 diabetes mellitus (T2-DM) was really fascinating, because of the idea of the capacity of the body to try adapt itself to adverse conditions by creating compensatory mechanisms of maintaining homeostasis: to overcome IR due to visceral adipose tissue excess, the pancreas secretes more insulin, which maximizes the entrance of glucose to the cells in order to avoid hyperglycemia and diabetes. IR could then be an endocrine model of the crucial importance of endocrine feedback physiology in the WOB. The problem with these adaptation mechanisms is that they have two main implications. The first is that there is a threshold for the compensation, and when that threshold is surpassed, adaptation fails. Physiology gives way to pathology; health becomes illness, in that case euglycemia becomes hyperglycemia and diabetes appears. Second, the steady state of IR is maintained at expense of an excess of insulin secretion by the pancreas (hyperinsulinism) and chronic hyperinsulinism can also be deleterious for the body.

In 1989, I read a wonderful publication of Kaplan about the deadly quartet (Kaplan 1989), where the author beautifully described the importance of predominance of upper-body weight along with hypertension, diabetes, and hypertriglyceridemia, to cardiovascular risk, even in the absence of significant overall obesity. That

article reminds to me an analogy with the Dumas classic about the three musketeers (Dumas 2007). Portus is the upper obesity, Atos is the hypertriglyceridemia, and Aramis is the hypertension. Later comes d'Artagnan to join them, as later comes T2-DM to join the triumvirate, which completes the deadly quartet.

When I joined the Endocrinology Department of the Portuguese Institute of Oncology of Oporto (*Instituto Português de Oncologia Francisco Gentil, IPOFG, Porto*) to work on endocrine oncology, I became very surprised to understand that two apparently unrelated fields had so much in common. As Marc Lippman stated in his article about Chaos Theory and a Career in Medicine (Lippman 2012) “I became torn between endocrinology and oncology, linking the former for the science and the latter for the sheer existential magnitude of the illness”. One has to recall at this time there was little if any relationship between the two specialities, and when I choose to work at the Portuguese Institute of Oncology, someone said that I was only going to treat diabetes of dying people and papillary thyroid carcinoma patients.

Then, Dr. Manuel Portocarrero, challenged me to study the relationship between T2-DM and breast cancer, and I decided to bring that subject to my Master Thesis. The more I studied in order to find where on earth there was an association between the two diseases, the more I was enthusiastic about the theme, as the first articles about IR and hyperinsulinism as the missing link between obesity, diabetes and breast cancer were emerging (Kaaks 1996). So, I realized that endocrinology and oncology were intrinsically linked. Apart from the deadly quartet risk for ischemic heart disease, ischemic cerebral disease and cerebral hemorrhagic disease, cancer was also a complication of obesity and diabetes – another deadly quartet!

PREAMBLE

After finishing my Master Degree in 2005, many questions were still waiting for answers. Another challenge was calling for me – to go on with the studies through a doctoral degree. In between, for personal reasons, professional life had to wait a few years. Meanwhile, what seemed to be a less productive phase, revealed to be a time for maturation of ideas and definition of what would be the next steps.

I was divided between the theme of the Master Thesis, which was about obesity, metabolic syndrome (MetS) and breast cancer, and my other passion in endocrine oncology, neuroendocrine neoplasia (NEN) a subject in which I invested so much energy in my hospital and my country in the last decade! Curiously, before 2005 these two areas of knowledge were somehow underestimated by the scientific community. In the first case because at that time, investigation on obesity, IR and cancer was scarce and limited to few authors and investigation of putative causes of cancer was focused on genetic and molecular biology fields. In the second case, because neuroendocrine neoplasia was considered a very rare, benign and a slow growing disease. In between, something has changed! The association between cancer and obesity became a hot topic in the literature. Also, scientific meetings and investigation in the field of neuroendocrine neoplasia registered an exponential rise, as it is actually the most increasing neoplasia in the world.

Then, the first question from my practical knowledge was obvious: why well differentiated neuroendocrine neoplasia's patients, even those with metastatic disease, are generally obese and stay in a good shape until late stages of the disease? They look so well that is hardly to believe they have a malignant tumor, most of the times already with liver metastasis! The second obvious question is almost a mathematical equation: if neuroendocrine neoplasia is on rise and recent publications confirm the association of many cancers with obesity and MetS, why not neuroendocrine neoplasia`s burden could be also related to

metabolic factors? And if that is to be proven, what are the mechanisms involved in the pathogenesis of neuroendocrine tumor`s development? The first thought that came to my mind was mammalian target of rapamycin (mTOR) pathway). The role of phosphoinositide 3-kinase/ serine/threonine kinase/ mTor (PI3K/Akt/mTor) pathway as a point of convergence between metabolism, nutrient status, growth factor signaling, and cellular proliferation is of extreme importance in understanding the relationships between metabolism and cancer, as in this particular case of neuroendocrine neoplasia.

The more I was reading about recent literature, the more a possible connection between the two conditions was becoming to make sense! Some of the organs that are involved in obesity, IR and T2-DM are the same generally affected by digestive neuroendocrine neoplasia: the gut, the pancreas and the liver.

In the beginning of the century, cancer was considered a fatality caused by genetic mutations of the cells. Nowadays we know that cancer is seldom hereditary and that risk factors (RFs) related to modern lifestyle in developed countries responsible for obesity and diabetes, are also the basis for neoplastic transformation of the cells.

Slowly, that idea of investigating obesity and MetS`s influence in the recent neuroendocrine neoplasia`s burden was coming to my mind. As a consequence, the need to study if that burden is somehow linked with three of the most important epidemics of the “civilized” world, namely obesity and diabetes, and indirectly cardiovascular disease (CVD) became a necessity!

ACKNOWLEDGEMENTS

I concluded my Master's Degree in 2005. In the preamble of Master Thesis, I wrote that in Portugal the hospital organization system does not privilege medical research, as medical doctors timetable is essentially focused on the clinical activity in detriment of investigation and teaching, that are the other 2 of the 3 pillars of the equilateral triangle that supports a tertiary care health unit.
Although the situation has changed a lot during these 15 years for basic research, clinical investigation for medical doctors is still of limited access, conditioned by the enormous amount of clinical activity that fulfill most part of their schedule.

I cannot forget that the final work that is presented now, was only possible with the help of all that joined to me in the voluntarism and enthusiasm needed to reach this final objective. So, I am infinitely grateful to those who believed in this idea and helped me to never giving up. I must mention some of those who contributed to finish this work, hopping I don`t miss anyone:

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Carlos, my shelter, to whom I can trust my ideas, doubts and insecurities with the certainty that I can continue to be as I am.

TABLE OF CONTENTS

ABSTRACT

Introduction

Obesity, T2-DM, CVD and cancer are four of the most prevalent non-communicable diseases (NCD), which are the major responsible for mortality, morbidity, and healthcare costs worldwide. Obesity and diabetes were demonstrated to be modifiable RFs for several malignancies. MetS, classically considered a cluster of cardiovascular RFs, was more recently also associated with cancer risk. Subclinical chronic inflammatory status that characterizes IR was appointed as the possible link between MetS and cancer.

GEP-NEN are relatively rare tumors whose incidence increased 720% in the last 30 years, being actually the second most prevalent neoplasia of the digestive system after colorectal cancer. Although research has enabled considerable advances in cancer genetics, molecular mechanisms and treatment of these tumors, true reasons and pathphysiological mechanisms underlying the recent tumor burden are unknown.

Aims

The main goal of the present thesis was to investigate whether well differentiated (WD) GEP-NEN`s burden could be influenced by modifiable RFs as obesity, hyperglycemia and MetS in a similar mode as observed for other neoplasia. We also aimed to study whether the individual components of MetS could influence WD GEP-NEN`s characteristics. In addition, immunohistochemistry studies in tumor tissues were conducted order to identify putative molecular signatures linking WD GEP-NEN and MetS, which could provide further insight into potential mechanisms for the association hypothesis.

Methods

The first step, as coordinator of the Neuroendocrine Tumors Study Group (GE-TNE) of the Portuguese Endocrinology, Diabetes and Metabolism Society (SPEDM) at the time, was to map the landscape of Neoendocrine Neoplasia in

Portugal. For that purpose a demographic and clinical characterization of the patients with neuroendocrine neoplasia attended in the main hospitals in the country was performed.

Then, we evaluated the association of WD GEP-NEN with the presence of MetS diagnostic criteria and individual components in a case-control study based in a cohort of patients followed at the Portuguese Institute of Oncology of Porto that were cross-matched with a control group with the same age, gender and origin derived from the Portuguese Metabolic Syndrome Study (PORMETS), a national survey of the prevalence of MetS in the Portuguese general population.

The next step was to investigate whether the presence of MetS or any of the individual components at the time of diagnosis, influenced the main charactheristics of the tumors, such as primary location, presence of hormonal secretion syndrome, World Health Organization (WHO) 2010 grading and disease extension.

At last, histochemical studies for markers of proliferation (Ki67), de-differentiation (Forkhead box protein M1, FOX M1), inflammation (interleukin-6, IL-6) and growth factors (insulin-like growth factor 1 receptor; IGF1R) on tumor histological sections of a cohort of operated patients were performed and correlated with the clinical findings.

Results

Our results showed that the national landscape of patients with GEP-NEN diagnosed in Portugal is not very different from the reality in other European countries, specially in South Europe. Also we stressed the need of skilled a multidisciplinary team in terciary hospitals which should aggregate the management of a rare disease as well as the importance of the presence of the trained endocrinologists in order to conduct an accurate characterization of the patients and the tumor, as the most efficient manner to define a correct treatment strategy.

We also demonstrated the association of MetS and individual components, such as abdominal obesity, high fasting plasma glucose (FPG) and triglycerides (TG) with WD GEP-NEN. The presence of MetS also influenced a better differenciation of the tumors and its extension, being more frequent in NET G1 versus NET G2 and metastatic versus localized and loco-regional disease.

Besides, our molecular studies demonstrated that although MetS did not influence FOXM1, IGF1R and IL-6 expression, IL-6 expression was influenced by the MetS feature low High Density Lipoprotein Cholesterol (HDL-c), and positively associated with disease progression in gastrointestinal NEN (GI-NEN).

Conclusion

In summary, the present results stress the potential influence of obesity and metabolic abnormalities in WD GEP-NEN´s risk and pathological characteristics. In addition, the research supports the role of the underlying inflammatory status that characterizes obesity, IR and MetS, as a common link between the two conditions.

These preliminary findings open a fascinating research field, where in a similar manner as observed for non-neuroendocrine neoplasia, WD GEP-NEN development could be influenced by modifiable RFs.

Overall, our findings unravel the unprecedent possibility that disease could be preventable, although they need to be confirmed in large multicentre studies.

LIST OF PUBLICATIONS

Paper 1[1] – Chapter 3.1

Santos AP, Vinagre J, Soares P, Claro I, Sanches AC, Gomes L, Fernandes I, Catarino AL, Preto J, Pereira BD, Marques AP, Rodrigues F, Amaral C, Rocha G,Mellidez JC, Simões H, Lopes JM, Bugalho MJ; On behalf of the NETs Study Group of the Portuguese Society of Endocrinology, Diabetes and Metabolism. Gastroenteropancreatic Neuroendocrine Neoplasia Characterization in Portugal: Results from the NETs Study Group of the Portuguese Society of Endocrinology,Diabetes and Metabolism. Int J Endocrinol. 2019 Aug 1;2019:4518742 (2018 impact factor 2.287).

Paper 2 – Chapter 3.2

Santos Ana P, Santos AC, Castro C, Raposo L, Pereira SS, Torres I, Henrique R, Cardoso H, Monteiro MP. Visceral Obesity and Metabolic Syndrome Are Associated with Well-Differentiated Gastroenteropancreatic Neuroendocrine Tumors. Cancers (Basel). 2018 Aug 27;10(9):293 (2018 impact factor 6.162).

Paper 3 – Chapter 3.3

Santos Ana P, Castro C, Antunes L, Henrique R, Cardoso MH, Monteiro MP. Disseminated Well-Differentiated Gastro-Entero-Pancreatic Tumors Are Associated with Metabolic Syndrome. J Clin Med. 2019 Sep 17;8(9):1479 (2018 impact factor 5.583).

Paper 4[2] - Chapter 3.4

Pereira SS, Pereira R, **Santos Ana P**, Costa MM, Morais T, Sampaio P, Machado B, Afonso LP, Henrique R, Monteiro MP. Higher IL-6 peri-tumoural expression is associated with gastro-intestinal neuroendocrine tumour progression. Pathology. 2019 Oct;51(6):593 (2018 impact factor 3.163).

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DECLARATION OF CONTRIBUTORS

The majority of the work carried out in this thesis was performed by the author. Any collaboration is described below.

Chapter 3.1:
All the authors were members of the Neuroendocrine Tumors Group of the Portuguese Society of Endocrinology, Diabetes and Metabolism at the time of the Study.
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ABBREVIATIONS

5-HIAA – 5-Hydroxyindoleacetic Acid

5-HT – 5-Hydroxitriptamin (serotonin)

^{18}F-FDG-PET – ^{18}F-fluorodeoxyglucose Positron Emission Tomography

^{68}Ga-PET – ^{68}Gallium-Position Emission Tomography

ACE – Angiotensin-Converting Enzyme

ADA – American Diabetes Association

AHA – American Heart Association

AICR – American Institute for Cancer Research

Akt – Serine/Threonine Kinase

AMPK – 5' Adenosine Monophosphate-Activated Protein Kinase

APCA – Anti-Parietal Cell Antibodies

ARA II – Angiotensin II Type Receptor Antagonists

ARIC – Atherosclerosis Risk in Communities

ATP III – Adult Treatment Panel III

bFGF – Basic Fibroblast Growth Factor

BMI – Body Mass Index

BP – Blood Pressure

BP-NEN – Broncho-Pulmonary Neuroendocrine Neoplasia

B-Raf – v-Raf Murine Sarcoma Viral Oncogene Homolog B

CAD – Coronary Artery Disease

CCE – Cancer Core Europe

CCK – Cholecystokinin

CD - Celiac Disease

CD8+ – CD8+ (cytotoxic) T Cells

CgA – Chromogranin A

CHANCES – Consortium on Health and Ageing: Network of Cohorts in Europe and the United States

CI – Confidence Interval

CPE – Cancer Prevention Europe

CRF – Chronic Renal Failure

CRP – C-Reative Protein

CT – Computerized Tomography

CVD – Cardiovascular Disease

DBP – Diastolic Blood Pressure

DFS – Disease Free Survival

DPP-4 – Dipeptidyl-peptidase-4

EASD – European Association for the Study of Diabetes

ECCC – European Compreensive Cancer Centers

ECL – Enterochromaffin-Like Cells

EMA – European Medicines Agency

ENETS – European Neuroendocrine Tumors Society

ERK – Extracellular-Signal-Regulated Kinase

FDA – Food and Drug Administration

FFA – Free Fatty Acids

FOX M1 – Forkhead Box Protein M1

FPG – Fasting Plasma Glucose

FPI – Fasting Plasma Insulin

GEN – Gastric Endocrine Neoplasia

GEP – Gastro-Entero-Pancreatic

GE-TNE – *Grupo de Estudos de Tumores Neuroendócrinos* (Neuroendocrine Tumor`s Study Group)

GI-NEN – Gastrointestinal Neuroendocrine Neoplasia

GIP – Glucose-dependent Insulinotropic Polypeptide

GLP-1 – Glucagon-like Peptide - 1

GLP-1ras – Glucagon-Like Peptide-1 Receptor Agonists

GPCR – G-Protein Coupled Receptors

GRS – Genetic Risk Score

GTT – Glucose Tolerance Test

HC – Hip Circumference

HDI – Human Development Indices

HDL-c – High Density Lipoprotein Cholesterol

H&E - Haematoxylin and Eosin

HER2 – Human Epidermal Growth Factor Receptor 2

HHV8 – Human Herpes Virus Type 8

HIV – Human Immunodeficiency Viruses

HOMA-IR – Homeostatic Model Assessment for Insulin Resistance

HP – Helicobacter Pylori

HPF – High Power Field

HPFS – Health Professional Follow-up Study

HPV – Human Papillomavirus Infection

HR – Hazard Ratio

HVB – Hepatitis B Virus

HVC – Hepatitis C Virus

IARC – International Agency for Research on Cancer

IAS – International Atherosclerosis Society

IASO – International Association for the Study of Obesity

IDF – International Diabetes Federation

IDFTFEP – International Diabetes Federation Task Force on Epidemiology and Prevention

IFG – Impaired Fasting Glucose

IGF1 – Insulin Growth Factor 1

IGF1R - Insulin Growth Factor 1 Receptor

IGT – Impaired Glucose Tolerance

IHD – Ischaemic Heart Disease

IL-2 – Interleukin-2

IL-6 – Interleukin-6

IPOFG – Portuguese Institute of Oncology Francisco Gentil (*Instituto Português de Oncologia Francisco Gentil*)

IR – Insulin Resistance

JDRF – Juvenile Diabetes Research Foundation

JIS – Joint Interim Statement

JNK – c-Jun N-Terminal Kinases

KPC – Kaiser Permanent Collaboration

LDL-c – Low Density Lipoprotein Cholesterol

LPS – Lipopplysaccharide

MAPK – Mitogen-activated Protein Kinase

MEN 1 - Multiple Endocrine Neoplasia Type 1

MetS – Metabolic Syndrome

MDR1 – Multidrug Resistance Mutation 1

MiNEN – Mixed Neuroendocrine-Non-Neuroendocrine Neoplasms

mTOR – Mammalian Target Of Rapamycin

mTORC 1 – Mammalian Target Of Rapamycin Complex 1

NAD – Nicotinamide Adenine Dinucleotide

NADH – Nicotinamide Adenosine Dinucleotide Hydride

NAFLD - Non-Alcoholic Fatty Liver Disease

NF-κB – Factor Nuclear Kappa B

NCD – Non-Communicable Diseases

NEC – Neuroendocrine Carcinoma

NEN – Neuroendocrine Neoplasia

NET – Neuroendocrine Tumors

NHANES – National Health and Nutrition Examination Survey

NHBLI – National Heart, Lung, and Blood Institute

NHL – Non-Hodgkin Lymphoma

NHS – Nurses´ Health Study

NIDDM – Non-Insulin-Dependent Diabetes Mellitus

nPOD – Network for Pancreatic Organ Donors with Diabetes

NRs – Nuclear Receptors

NSCLC – Non-Small Cells Lung Cancer

ORIGIN – International Outcome Reduction with Initial Glargine Intervention

OS – Overall Survival

PAF – Population Attributable Fracction

PanNEN – Pancreatic Neuroendocrine Neoplasia

PE – Physical Exercise

PI3K – Phosphoinositide 3-kinase

PIGF – Phosphatidylinositol Glycan Anchor Biosynthesis Class F

PORMETs – Portuguese Metabolic Syndrome Study

PP – Pancreatic Polypeptide

PPAR – Peroxisome Proliferator-Activated Receptors

PT – Primary Tumor

PYY – Peptide Tyrosine Tyrosine

Ras – Rat Sarcoma Vírus

REDD1 - Regulated In Development And DNA Damage Responses 1

RF – Risk Factor

RORENO – North Oncologic Registry *(Registo Oncologico do Norte)*

ROS – Reactive Oxygen Species

RR – Relative Ratio

RTK – Receptor Tyrosine Kinase

SA – Somatostatin Analogues

SBP – Systolic Blood Pressure

SD – Standard Deviation

SDI – Sociodemographic Index

SEER – Surveillance, Epidemiology and End Results

SERT – Serotonin Transporters

SGLT2 – Sodium-Glucose Co-Transporter-2

SI-NEN – Small Intestine Neuroendocrine Neoplasia

SOAS – Sleep Obstructive Apnea Syndrome

SPEDM – Portuguese Society of Endocrinology, Diabetes and Metabolism (*Sociedade Portuguesa de Endocrinologia, Diabetes e Metabolismo*)

SSTR – Somatostatin Receptor

T1-GEN – Type 1 Gastric Endocrine Neoplasia

T2-DM – Type 2 Diabetes Mellitus

TAM – Tumor Associated-Machrophages

TC – Total Cholesterol

TG – Triglycerides

TGF – Transforming Growth Factor

TKI – Tirosine Kinase Inhibitors

TKR – Tirosine Kinase Receptors

TNF-α – Tumor Necrosis Factor Alpha

UV – Ultraviolet

VAT – Visceral Adipose issue

VEGF – Vascular Endothelial Growth Factor

WC – Waist Circumference

WCRF – World Cancer Research Fund

WHF – World Heart Federation

WHO – World Health Organization

WHR – Waist-To-Hip Ratio

Chapter 1 - INTRODUCTION

"Cancer is a chronic systemic disease with local manifestations like arteriosclerosis, which is also systemic and manifested solely by its local manifestations, e.g. stroke and myocardial infarction. In the same way as treatment of an ailing heart does not cure the underlying arteriosclerosis, tumor removal does not cure cancer, as it is 'metabolically' systemic. It is proposed here that carcinogens deplete a vital substance and induce a metabolic deficiency that ends in cachexia. In order to survive, the organism grows a protective organ - the tumor - that replenishes the missing substance. During the preclinical phase of cancer, deficiency is slight and compensated only by a minute tumor. With time, it gets worse and the tumor has to grow more and more in order to make up for the loss, causing pain and secondary damage to vital functions. The patient seeks help and the disease starts its clinical course. When deficiency worsens, the patient becomes cachectic and dies."

….in New Cancer Hypothesis G. ZAJICEK. (Zajicek 1996)

1.1 The XXI`s Century Epidemics

In the beginning of XX century, infectious diseases were the major causes of dead in the world. Even at the present, in some low-income countries these are still the predominant cause of death in most of the population at young ages (Remais, Zeng et al. 2013). In the era of antibiotics, developed countries still have a high prevalence of infectious diseases, mainly viral diseases like HIV (human immunodeficiency viruses) and HPV (Human papillomavirus infection). However, even these infection conditions are no longer so deadly as initially, due to the widespread use of vaccines and new antiviral agents, which have turned these conditions into chronic diseases.

In the end of the last century a change of paradigm occured. The adoption of a western lifestyle was accompanied by an increase of NCDs. In 2016, 40·5 million (71%) of the 56·9 million worldwide deaths were estimated to result from NCDs. Among these, 32·2 million NCDs deaths (80%) were estimated to be due to cancers, CVD, chronic respiratory diseases, and diabetes, and another 8·3 million (20%) to other NCDs. Globally, the lowest risks of NCD mortality were observed in high income countries in Asia-Pacific, Western Europe, Australia, and Canada. The highest risk of dying from NCDs was observed in low-income

and middle-income countries, especially in sub-Saharan Africa, and specifically for men, in Central Asia and Eastern Europe (collaborators 2018).

Obesity was identified to be the common link between the first four most frequent NCDs. Causes for obesity are complex and still under debate. Surely, a change in lifestyle pattern related with an increase in rich sugar and fat diet combined with sedentary habits associated with technology development, organization of the cities and sitting working hours, played a major contribution (Swinburn, Caterson et al. 2004, Panahi and Tremblay 2018).

According to global survey of obesity in 195 countries performed in 2015, 604 million adults and 108 million children were obese. Since 1980, prevalence of obesity doubled in 73 countries and increased in most other countries. Of even greater concern was that the rate of increase was even greater in childhood (Collaborators, Afshin et al. 2017). Although at the beginning, these were the population patterns of high-income countries of the western so called “civilized” world, since the end of the XX century emergent countries, such as China and India and also developing sub-Saharan countries, also became increasingly affected by these conditions in result of the adoption of western lifestyles accompaning the growth patterns of economies (Kelly, Yang et al. 2008). These social modifications were responsible for the so called “nutrition transition” phenomenon, characterized by a change in eating habits with high energy and fat diets and low physical activity in low income countries described by Poppkin in 2001 (Popkin 2001). In these countries a nutrition paradox often occurs, with coexistence of undernutrition and obesity, particularly in the urban setting and sometimes in the same family . Consequently, epidemiological evidence suggests that the prevalence of overweight and obesity is increasing in many developed and developing countries and that these changes are likely to continue over the next few decades (Prentice 2006). So, these conditions will be a major public health problem the world is going to face in the near future.

In 2002, me and Couto studied the alimentary habits of a small rural community of Santiago de Cabo Verde (Couto and Santos 2009). In this cohort, the authors realized the role of nutrition transition, where “higher” income families had begun to change their lifestyle habits from a diet based on slow absorption

carbohydrates and vegetable proteins to a relatively high energy diet, based on rapid absorption carbohydrates, high sugar drinks and more animal proteins. The authors also found that the “higher” income families had excess of weight, including the teacher`s and the local market owner who had a more sedentary lifestyle since they did not need to walk several kilometres carrying water and collecting firewood.

Portuguese statistics from 2015 (Gaio, Antunes et al. 2018) revealed that the estimated national prevalence of overweight (including obesity) was 38.9% (95%CI: 36.9-41.1) and the prevalence of obesity was 28.7% (95%CI: 26.8-30.6). There was a higher prevalence of overweight among males, but the prevalence of obesity was higher among females; the elderly and individuals with the lowest level of education were the most affected. Although excess of weight remained stable (39%), obesity has duplicated from 14.2% to 28.7%, when compared with results of 2003-2005 (do Carmo, Dos Santos et al. 2008), thus the prevalence of obesity in Portugal is one of the highest in Europe. In Portugal, childhood obesity is also a concern. A Portuguese survey published in 2018 revealed that the prevalence of overweight among children was 21.9% (18.9 – 25.0), while obesity prevalence was 6.1% (4.2 – 8.0) (Rodrigues, Padez et al. 2018).

T2-DM is nowadays one of the greatest public health problems societies must face. As the majority of the most frequent NCDs, T2-DM is directly related to obesity. It was estimated that in 2017 there were 451 million people between 18-99 years with diabetes worldwide, half these with undiagnosed disease (Cho, Shaw et al. 2018). Impaired glucose tolerance (IGT) was present in 374 million and 5 million died because of causes attributable to diabetes (Cho, Shaw et al. 2018). In 2030, T2-DM prevalence is expected to rise by 32% in Europe and 72% in North America relatively to 2000 (Shaw, Sicree et al. 2010) and by 2045 it is expected to reach 693 million individuals (Cho, Shaw et al. 2018). This problem is even worse in developing countries, such as in sub-Saharan Africa, India and South America where an increase in prevalence of more than 150% is expected (Hossain, Kawar et al. 2007). The costs associated with diabetes in terms of morbidity and mortality are enormous. For example in USA, direct and indirect costs related to diabetes in 2017 were estimated to be 850 billion dollars (Whiting, Guariguata et al. 2011).

Again, Portugal follows the trends. Statistics from the *Observatório Nacional da Diabetes* relative to 2015 *(Observatório 2016)* show that estimated diabetes prevalence in the Portuguese population between 20-79 years was 13.3% (one million of Portuguese), with a growth rate of 13.5% and, what is more worrisome, 44% of the population being undiagnosed.If one consider intermediate hyperglycaemia which means impaired fasting glucose (IFG) and IGT, the figures are even higher, with nearly 3.5 million affected.

MetS, also known as X-syndrome is a constellation of CVD RFs that has been defined according to slightly different criteria by various organizations (Saklayen 2018). MetS is characterized by abdominal obesity, IR, hypertension, and hyperlipidaemia (Alberti, Eckel et al. 2009), that feeds into the spread of diseases like T2-DM, coronary diseases, stroke, and other disabilities. The total costs related to MetS, including the cost of health care and loss of potential economic activity is likely to ascend to trillions of dollars (Saklayen 2018). There is a consensus about the importance of fat distribution in CVD risk, with abdominal obesity being associated with more adverse metabolic profile and consequently negative impact on CVD risk, even with normal body mass index (BMI) (Despres 2012). Statistics from the PORMETS (Raposo, Severo et al. 2017) showed that the prevalence rates of MetS in a sample of Portuguese adults were 36.5%, 49.6%, and 43.1%, using the Adult Treatment Panel III (ATP-III), International Diabetes Federation (IDF) and Joint Interim Statement (JIS) definitions, respectively. The most prevalent feature of MetS in PORMETS was high blood pressure (BP) (64.3%) and the lowest was high fasting glucose (24.9%). Evaluation of the prevalence of MetS in children was also addressed and again children were affected; 21.9% (18.6 – 25.0) had a waist-to-hip ratio (WHR) ≥ 0.50 (Rodrigues, Padez et al. 2018).

CVD is another frequent NCD that is intrinsically linked to obesity and other related RFs, such as hypertension and dyslipidaemia. Many advances in the treatment of the disease have been made in both prevention and treatment. These include the widespread use of statins and other progresses, as precipitous declines in cigarette smoking, improvements in hypertension treatment and control and the development and timely use of thrombolysis and stents in acute coronary syndrome to limit or prevent infarction (Mensah, Wei et al. 2017). Some

countries with a high sociodemographic index (SDI) had dramatically decreased the mortality associated with CVD, mainly ischaemic heart disease (IHD) and stroke, because of the programmes focused on implementing a healthy lifestyle. Anyway, there is a wide variation on regional prevalence of CVD, according not only to exposure to modifiable RFs but also to access to health care interventions and again the rising of CVD in developing world contributes to the plateau shaped curve observed in global mortality (Roth, Johnson et al. 2017).

Cancer is also one of the world's epidemics of the 21st century in the so-called civilized world and is expected to rank as the leading cause of death and the single most important barrier to increasing life expectancy in every country of the world. According to 2015 estimates from WHO, cancer is the first or second leading cause of death before age 70 years in 91 of 172 countries, and it ranks third or fourth in an additional 22 (Bray, Ferlay et al. 2018). Cancer cases increased 28% from 2006 to 2016, the smallest increase was in high income countries (Sung, Siegel et al. 2019).

Although population ageing and population increase contribution was nearly 30%, other factors must influence this cancer incidence and mortality epidemics (Global Burden of Disease Cancer, Fitzmaurice et al. 2018). Despite great efforts on screening and the advent of new drugs for treatment, incidence and mortality of cancer is rapidly growing all over world. This phenomenon cannot be solely explained by the population aging and by the decline in mortality due to CVD. Besides, the early onset cancer diagnosis in young adults between 25-49 years is increasing, namely in colorectal cancer (Siegel, Miller et al. 2017). Again, an increasing magnitude is observed in emerging economies where a 60% increase in new cases is estimated by 2030, accompanied by a cancer transition paradigm from infection-related and poverty-related cancers to those cancers that were already highly frequent in the most developed countries, (Bray, Ferlay et al. 2018).

Data from RORENO (Cancer Registry of Northern Portugal) (https://www.ipoporto.pt/dev/wp-content/uploads/2013/03/Publ_Projecoes.pdf) concerning 2012, estimated an increase of 27.3% (21.9-32.6%) cases of non-melanoma malignant tumors relative to 2008 for 15 primaries tumors with the

exception of cervix cancer, which shows a decrement in age-standardized rate between 1994 and 2008. Projections to 2020 show a global tendency for an increase in incidence in non-melanoma malignancies (Fig. 1).

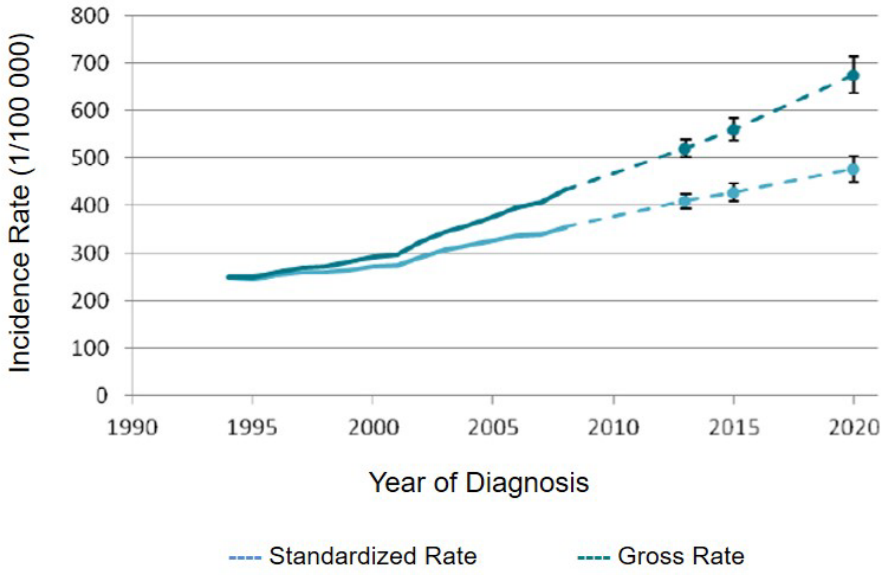

Figure 1. Age-standardized rate tendency of incidence of non-melanoma malignancies and projections to 2020 *(Adapted from RORENO, 2013)*

Cancer was previously considered a fatality caused by unexplained mutations in putative genes, and the unlucky affected individuals had to accept this hazard which could not have been prevented. All cancer research was focused on screening and treatment, as the only way to decrease mortality was early detection and therapeutic intervention. Prevention was limited to lung cancer and infection-related cancers, through smoke cessation and promotion of protective actions of transmission and vaccination. In the end of 20th century, a new paradigm began to gain consistence. Alongside with genetic alterations, environment changes characteristic of western lifestyle were fundamental in the development of many cancers. Statistics all over the world showed the influence of modifiable RFs on the development of most cancers. In 2014, at the United States an estimated 42.0% of all incident cancers (659,640 of 1570,975 cancers, excluding non-melanoma skin cancers) and 45.1% of cancer deaths (265,150 of

587,521 deaths) were attributable to evaluated RFs. Actually, the main modifiable RF is active and passive cigarette smoking (19% of cases; 28.8% of deaths), but other RFs such as alcohol intake, ultraviolet (UV) radiation exposure; low dietary calcium and infection with Helicobacter Pylori (HP), hepatitis B virus (HBV), hepatitis C virus (HCV), human herpes virus type 8 (HHV8), HIV or HPV also have an important role (Siegel, Miller et al. 2017, Islami, Goding Sauer et al. 2018). The remaining and large proportion of modifiable RFs are all related to obesity: excess body weight; consumption of red and processed meat; low consumption of fruits and vegetables and dietary fibber and physical inactivity.

1.2 Obesity and Cancer

An historical paper published by Tannenbaum (Tannenbaum 1940) described for the first time the association between weight and the incidence of cancer, based on insurance company statistics and animal models. In the late XXth and beginning of XXIth century, studies on the relationship between BMI and cancer were published by few authors like Calle (Calle, Rodriguez et al. 2003) and Stoll (Stoll 1996). At the same time, papers from Schapira first described the association between visceral obesity and breast cancer (Schapira, Kumar et al. 1990).

Since then, obesity and cancer crosstalk were object of an intensive curiosity by the scientific community and nowadays there are hundreds of studies focused on this theme, which is also an hot topic in several meetings in the field of obesity and oncology.

One of the most important epidemiological contribution was a 2008 publication by Renehan, a meta-analysis which searched for studies on obesity and cancer and concluded that a 5 kg/m^2 increase in BMI was strongly associated with oesophageal, thyroid, colon and renal cancer in men and endometrial, gallbladder, oesophageal and renal cancer in women. A weak association was found concerning retal cancer and melanoma in men and post-menopausal breast, pancreas, thyroid and colon cancer in women. In both sexes, leukaemia,

multiple myeloma and non-Hodgkin lymphoma were also associated with BMI (Renehan, Roberts et al. 2008).

In 2014, Bhaskaran updated the epidemiological data in almost 170 million UK individuals and confirmed the association between obesity and 17/22 cancers. A 5 kg/m^2 increase in BMI was associated with increased risk of the uterus, gallbladder, kidney, cervix, thyroid and leukaemia. BMI was positively associated with liver, colon, ovarian, and post-menopausal breast cancer (all $p<0.0001$). Conclusions also pointed that a 1 kg/m² population-wide increase in BMI would result in 3790 additional annual UK patients developing one of the ten cancers related to BMI (Bhaskaran, Douglas et al. 2014).

A 2017 review of the meta-analysis published is not so convincing about this topic (Kyrgiou, Kalliala et al. 2017). It emphasised the heterogenicity of the published studies and concludes that evidence is only strong in 11 cancers, namely digestive and hormone dependant cancers.

Anyway, all of the most recent publications (Colditz and Peterson 2018, Avgerinos, Spyrou et al. 2019) about this subject and involving the International Agency for Research on Cancer (IARC) group and also the World Cancer Research Fund / American Institute for Cancer Research (WCRF/AICR) are consensual about the influence, with different levels of evidence, of overweight and obesity on 13 different types of cancers, including breast, colorectal, endometrial, oesophageal adenocarcinoma, gallbladder, gastric, kidney (renal cell), liver, multiple myeloma, ovarian, pancreas and thyroid (Table I).

Table I. Epidemiological Evidence Associating Overwehight/Obesity and Cancer by Level of Evidence and Strength of Relative Risk Increase for Overwheight/Obesity in Comparison to Normal-Range Body Mass Index Defined by WHO as Synopsized by the IARC Working Group In 2017

Evidence level	**Strengh of Relative Risk Increase for Obesity and Cancer Risk**		
	High (RR increase ≥3)	Modest (RR increase: 1.50-2.99)	Little (RR increase: ≥1.0<1.49)
Convincing / Sufficient	Endometrial Adenocarcinoma	Renal Adenocarcinoma	Colorectal Cancer
	Esophageal Adenocarcinoma	Hepatocellular Cancer	Postmenopausal Breast Cancer
		Pancreatic Adenocarcinoma	Gallbladder cancer
		Gastric Cardia Cancer	Ovarian cancer
		Multiple Myeloma	Thyroid cancer
		Meningioma	
Limited		Advanced Prostate Cancer	
		Male Breast Cancer	
		Diffuse Large T-Lymphoma	

Adapted from Avgerinos, 2019

The burden of obesity-related cancers worldwide parallels the distribution of the other non-communicable diseases. In 2012, 3·6% of all new cancer cases in adults (aged 30 years and older after the 10-year lag period) were attributable to high BMI (Arnold, Pandeya et al. 2015), especially in countries with very high and high distribution of human development indices (HDI). Projections to 2030 state that if nothing is done, overweight and obesity could surpass smoking as a significant preventable cause of cancer (Ligibel, Alfano et al. 2014). This problem is even more challenging in developing world, as the 2030 projections estimate an almost 150% incidence increase in obesity (Hossain, Kawar et al. 2007), which permits to infer a rise in obesity-related cancers in those countries, which must be able to face a new epidemic .

In 1996, Stoll BA stated that the distribution of adiposity was better than BMI as a risk marker for pre and post-menopausal breast cancer (Stoll 1996). Also, in the 90s, Schapira (Schapira, Kumar et al. 1990, Schapira, Clark et al. 1994) and Huang (Huang, Willett et al. 1999) among others, published the first studies on the association of waist circumference (WC), skin fold measures and WHR, and visceral obesity measured by CT,with breast cancer risk, independently of BMI.

A meta-analysis from seven prospective cohorts of Europe participating in the CHANCES (Consortium on Health and Ageing: Network of Cohorts in Europe and the United States) was published in 2017 (Freisling, Arnold et al. 2017). Besides BMI, the study evaluated the association of WC, hip circumference (HC) and WHR with obesity-related cancers in general and also with site specific neoplasia, like colorectal and post-menopausal breast cancer. The authors concluded that all measures were comparable in identifying positive associations with obesity-related cancers and colorectal cancer. For post-menopausal breast cancer, women never exposed to hormone therapy showed an almost 20% increase risk per standard deviation (sd) when compared to ever users.

More recently, a 2019 prospective study from Canada including 26 607 participants (Barberio, Alareeki et al. 2019), demonstrated the importance of central body fatness as a stronger predictor of cancer risk than overall body size, and this included colon and haematological cancer and non-Hodgkin lymphoma (NHL) in men and endometrial and post-menopausal breast cancer in women. Computed Tomography (CT) is the best way to direct quantification of visceral adipose tissue (VAT), although in clinical practice WC and WHR are indirect indicators of visceral adiposity. Visceral obesity as a RF for cancer cannot be underestimated, as lack of assessment can lead to obesity status misclassification and result in potential biases on the association between obesity/overweight and cancer towards to null effect (Allott and Hursting 2015). At least for for breast and colorectal cancer, there is enough evidence of the association of WC and WHR as a indicator of risk.

Another interesting aspect is the influence of the age of weight gain occurence on cancer risk (Stoll 1999, Agnoli, Grioni et al. 2015, Keum, Greenwood et al. 2015, Taghizadeh, Boezen et al. 2015). Publications from the late 1990s (Stoll 1999) and early 2000s already described the relationship between body weight at birth (Leong, Mignone et al. 2003) as well as the age at which excessive weight gain occured on breast cancer risk (Stoll 1995). At that time, Stoll stated that “experimental evidence suggests that the susceptibility of mammary tissue to carcinogenesis is greatest in early adultlife, and multiple studies show that a history of weight gain in early adult life is associated with increased breast cancer risk in western women”.

Although the association of obesity with cancer survival is not yet established because of multiple interferences in the study results, it also seems to be linked to poor outcomes and mortality in cancer patients (Demark-Wahnefried, Platz et al. 2012). A study published already in 2006 concluded that a BMI greater than 35.0 kg/m at diagnosis was associated with an increased risk of disease recurrence and death from colon cancer (Dignam, Polite et al. 2006). One year later, similar results were found in prostate cancer by Gong et al. (Gong, Agalliu et al. 2007), which observed that obesity at the time of diagnosis was associated with increased risk of prostate cancer metastasis and death. The increased risk of prostate cancer death or metastasis associated with obesity was independent of key clinical prognostic factors at diagnosis. An historical study from 2008, showed an increased mortality by cancer caused by adiposity over a mean follow-up of 9.7 years (Pischon, Boeing et al. 2008). Moreover, this was one of the first studies demonstrating an increased mortality associated with abdominal adiposity, independently of BMI ($p<0.001$). Relative risks (RR) among men and women in the highest quintile of WC were 2.05 (95% CI, 1.80 to 2.33) and 1.78 (95% CI, 1.56 to 2.04), respectively, and in the highest quintile of WHR, the RR were 1.68 (95% CI, 1.53 to 1.84) and 1.51 (95% CI, 1.37 to 1.66), respectively. A study published in 2010 by Balentine concluded that intra-abdominal fat was a good predictor of survival in pancreatic exocrine cancer (Balentine, Enriquez et al. 2010). Patients on the second quartile of abdominal fat measured by CT, had double risk of death (HR 4,018 95% CI 1.099-14.687; $p<0.035$). A meta-analysis from six large breast cancer case-cohorts involving 36 210 individuals found a causal effect of BMI on reduced breast cancer survival for estrogen receptor (ER) positive cases [(HR = 1.11 per one-unit increment of genetic risk score (GRS), 95% CI 1.01-1.22, $p = 0.03$)] (Guo, Burgess et al. 2017).

In 2014, the population attributable fracction (PAF) deaths attributable directly or indirectly to overweight/obesity were similar in both sexes (5.7%, 17560 in men; 7.4%, 20 690 in women), namely endometrial, gallbladder, liver, kidney, oesophagus, pancreas, stomach, breast, thyroid, multiple myeloma, colorectal and ovary (Islami, Goding Sauer et al. 2018).

1.3 Metabolic Syndrome, Type 2 Diabetes Mellitus and Cancer

Obesity, hypertension, dyslipidaemia and T2-DM are not only CVD RFs, but also represent risks for non-alcoholic fatty liver disease (NAFLD), Alzheimer disease and cancer.

MetS is a constellation of factors that traditionally increase the risk of CVD and have a greater chance of occurring together than alone. Despite MetS definition being somehow artificial and controversial, proved to be very useful in clinical practice. In order to unify the diagnosis criteria and overcome the previously existing heterogeneity in MetS definition, a decade ago, the International Diabetes Federation (IDF) Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute (NHBLI); American Heart Association (AHA); World Heart Federation (WHF); International Atherosclerosis Society (IAS); and International Association for the Study of Obesity (IASO), agreed on a Joint Interim Statement (JIS) towards harmonizing the MetS definition (Alberti, Eckel et al. 2009) (Table II).

Table II. Criteria for Clinical Diagnosis of the Metabolic Syndrome (≥ 3 criteria)

Elevated waist circumference[i]	≥ 102 cm (men); ≥ 88 cm (woman)
Elevated triglycerides (drug treatment for elevated triglycerides an alternate indicator[ii]	≥ 150 mg/dL
Reduced HDL-c (drug treatmentt for reduced HDL-c is an alternate indicator[ii])	< 40 mg/dL (men); < 50 mg/dL (women)
Elevated Blood Pressure (anti-hypertensive drug treatment in a patient with history of hypertention is an alternative indicator)	Systolic ≥ 130 and /or diastolic ≥ 85 mmHg
Elevated fasting glucose[iii] (drug treatment for elevated glucose is an alternative indicator)	≥ 100 mg/dL

[i]For European people is recommended to use either IDF or AHA/NHLBI (ATP III) cut points until more data available

[ii]The most commonly used drugs for elevated triglycerides and reduced HDL-c are nicotinic acid and fibrates. A patient taking one of these drugs can be presumed to have elevated triglycerides and reduced HDL-c. High dose ω-2 fatty acids presumes high triglycerides.

[iii]Most patients with type 2 diabetes mellitus will have the metabolic syndrome by the proposed criteria

Adapted from Alberti, Eckel et al. 2009

However, it should be emphasized that the MetS definition does not provide an absolute CV risk indicator, since many other contributing RFs, such as age, sex, cigarette smoking, and low-density lipoprotein cholesterol (LDL-c) levels are not considered. Anyhow, the impact for CVD incidence and mortality is high, since individuals that meet MetS criteria have twice the risk of developing CVD over the next 5 to 10 years, when compared to individuals without the same criteria. Additionally, MetS confers a 5-fold increase in risk for T2-DM (Kaur 2014).

A recent meta-analysis of MetS related mortality revealed that RR of all-cause and CV mortality was 1.23 (95%CI 1.15-1.32) and 1.34 (95%CI 1.11-1.39), respectively. Additionally, some of the MetS individual components also seem to influence mortality, namely high waist circumference, high FPG and low high density lipoprotein cholesterol (HDL-c). On the contrary, in elderly individuals, MetS was suggested to be protective, which could be partially explained by biases derived from malnutrition, immunocompromised function and frailty (Ju, Lee et al. 2017).

The PORMETS study allowed to evaluate the prevalence of MetS in the Portuguese population (Raposo, Severo et al. 2017). The MetS prevalence in a representative sample of the Portuguese adult population were 36.5%, 49.6%, and 43.1%, according to the different definitions that were used, which included the ATP-III, IDF and JIS, respectively. Moreover, MetS prevalence was significantly higher in non-urban than in urban populations ($p = 0.001$), in women ($p<0.001$) and older individuals ($p<0.001$), as well as, in those who reported being housewives ($p = 0.010$), retired ($p = 0.046$) or unemployed ($p = 0.024$).

In my MasterThesis work, a case-control study compared treatment naïve pre and post-menopausal women with breast cancer (n=47) to healthy women matched for age and menopausal status, and identified MetS ($p=0.003$), past history of foetal macrosomia (0.008), hypertension ($p=0.040$) and low HDL-c ($p=0.049$) as RFs for breast cancer. Furthermore, the risk of MetS duplicated in the group of sedentary patients ($p=0.05$). Besides, the risk of breast cancer was also significantly higher in women with BMI in the overweight/obesity range, elevated waist/hip ratio, elevated LDL-c (≥160 mg/dL) and abnormal Glucose Tolerance Test (GTT). In this study, traditional RFs for breast cancer (alcohol,

menarche < 12 y, menopause > 55 y, nulliparity, age of first birth >30 y and family history of breast cancer) were not found to be associated with an increased risk with the sole exception of menarche age <12 years (p=0.008) (Santos 2004). Although at that time, the risk association of obesity and breast cancer risk had been recently identified, the evidence on MetS as a RF for breast cancer were still scarce (Sinagra, Amato et al. 2002).

Since then, great advances were made to the point that MetS and some individual components are well recognized RFs for several cancers, breast and colorectal cancer included. Indeed, there is now sufficient evidence to state that not only MetS is a RF for cancer but also are MetS individual components, such as central obesity, hyperglycaemia, dyslipidaemia and hypertension, independently of BMI (Uzunlulu, Telci Caklili et al. 2016).

In an Italian patient cohort, post-menopausal but not pre-menopausal women with MetS were identified to have an increased risk for breast cancer (HR 1.80, 95%CI 1.22-2.65 vs HR 0.71, 95%CI 0.43-1.16) (Agnoli, Grioni et al. 2015). Elevated fasting plasma glucose (FPG) was also identified as a RF for both pre and post-menopausal breast cancer (HR 1.47 IC 95% 1.13-1.91; HR 1.89 IC95% 1.29-2.77). In 2012 Esposito et. al. published one of the first meta-analysis about MetS and the risk of cancer (Esposito, Chiodini et al. 2012). Although there was a great heterogeneity between the different studies, including the use of different MetS definition criteria, it was possible to conclude that MetS was associated with an increased risk of liver, colon, colorectal, pancreas, thyroid, rectal, bladder and prostate cancer in men and endometrium, pancreas, post-menopausal breast cancer, rectal, liver, colorectal, colon and ovary in women.

Additionally, these findings stressed the importance of considering the risk of cancer in individuals with MetS, even in the absence of obesity and diabetes, since abdominal obesity and IFG abnormalities were identified to be independent RFs for cancer as well. Indeed, elevated FPG was found to be independently associated with cancer risk (HR 1.20; 95% CI 1.03–1.39 in male, 1.28; 95% CI 1.08–1.53 in female) (Rapp, Schroeder et al. 2006). The strongest association was observed in hepatocellular cancer in men (HR 4.58; 95% CI 1.81–11.62) in a population-based study including 140 000 adults (63 585 males, 77 228 female)

followed for an average of 8.4 years. A publication from Taiwan, observed a linear correlation between plasma glucose and pancreatic cancer risk, even in pre diabetes (Liao, Tu et al. 2015). Every 0.56 mmol/L increase in FPG was associated with a 14% increase in the rate of pancreatic cancer. In 2008, the Atherosclerosis Risk in Communities (ARIC) Study (Kucharska-Newton, Rosamond et al. 2008), concluded that after adjusting for age, race, body mass index, smoking, and reproductive variables, there was an association of low baseline HDL-c (<50 mg/dL) with incident breast cancer in the total sample (HR 1.08 [95% confidence interval (CI), 0.84–1.40]) and a modest association (HR 1.67 95% CI, 1.06–2.63) among women who were premenopausal at baseline. The same group found an association between low HDL-c and lung cancer (Kucharska-Newton, Rosamond et al. 2008). Higher risk related to low HDL-c was also found in hematologic cancer (Shor, Wainstein et al. 2007) and high TG had been associated with prostate cancer risk (Wuermli, Joerger et al. 2005). Association of TG with breast cancer was already described in late 90`s by Goodwin et. al. (Goodwin, Boyd et al. 1997).

Besides being recognized as a RF for cancer incidence, MetS also seems to affect cancer recurrence and mortality. MetS was identified in 20.7% of 1069 patients with non-metastatic colorectal cancer, in a chinese prospective study (You, Liu et al. 2015). During a mean period of 59.6 months follow-up, the presence of MetS was an independent RF for disease free survival (DFS) (HR = 0.733, 95%CI 0.545–0.987, p = 0.041), but not for overall survival (OS) (p = 0.118) (You, Liu et al. 2015). A very recent meta-analysis concludes that the presence of Mets affects survival (Hu, Zhang et al. 2019). The presence of MetS significantly influenced digestive tract cancer survival in prospective studies (HR: 1.64, 95% CI: 1.18 to 2.28), in studies involving postsurgical patients (HR: 1.42, 95% CI: 1.06 to 1.92), and in studies assessing cancer-specific survival (HR: 1.91, 95% CI: 1.45 to 2.52). In a Japanese cohort with a mean 18.5 years follow-up, MetS was positively associated with cancer mortality in women (HR 1.69; 95%CI 1.21–2.36), but not in men (HR 1.21; 95%CI 0.90–1.62). Additionally, MetS was associated with a high risk of colorectal (HR 3.48; 95%CI 1.68–7.22) and breast cancer deaths in women (HR 11.90; 95%CI 2.25–62.84) (Watanabe, Kakehi et al. 2019). Asians studies are important as an increase of cancer

incidence and deaths is registered, as much as a western lifestyle modification is being adopted with consequent increase of obesity and associated metabolic disturbances.

Together and intrinsically related to obesity, T2-DM is a highly prevalent NCD worldwide with great impact on health due to long term consequences. CVD, cerebrovascular disease, chronic renal failure (CRF) and diabetic foot are well known devastating consequences of T2-DM since many decades ago. Evidence on the association of T2-DM and cancer is more recent, still precludes major challenges to healthcare systems in the future to come, as the estimated obesity and diabetes burden for the next decades also predict a tremendous rise in the incidence of cancer. The expected increments of life expectancy in result of implementation of healthcare policies and therapeutic advances aimed to reduce mortality from traditional T2-DM complications, will inevitably lead to an increase in cancer mortality.

To the extent that the growing evidence on the relationship between diabetes and cancer has led the American Association of Diabetes (ADA) to elaborate a consensus statement in 2010 (Giovannucci, Harlan et al. 2010). This document is aimed to address for four main questions: i) the association between diabetes and cancer incidence or prognosis, ii) RFs common to both diabetes and cancer, iii) biologic links between diabetes and cancer risk, and iv) whether diabetes treatments influence the risk of cancer or cancer prognosis. Non-modifiable factors associating T2-DM and cancer are gender (men), age (older) and ethnicity (African Americans and non-Hispanic whites in USA). Modifiable factors include overweight, obesity and weight change, diet, sedentary lifestyle, tobacco and alcohol.

A 2018 publication from Pearson-Stuttard et. al. about both the influence of both obesity and diabetes on cancer relatively to 2012 concluded that 5·6% of all incident cancers were attributable to the combined effects of diabetes and high BMI as independent RFs, corresponding to 792 600 new cases. Individually, high BMI (544 300 cases) was responsible for twice as many cancer cases as diabetes (280 100 cases) and 26·1% of diabetes-related cancers (77 000 new cases) and 31·9% of high BMI-related cancers (174 040 new cases) were

attributable to increases in the prevalence of these RFs from 1980 to 2002 (Pearson-Stuttard, Zhou et al. 2018).

Cancer treatment with some drugs used in chemotherapy like 5-fluorouracil and carboplatin/paclitaxel, glucocorticoids and androgen-deprivation therapy can induce or worsen hyperglycaemia (Jacob and Chowdhury 2015).

Moreover, experimental and animal studies have shown that depending on glucose levels the cytotoxicity of chemotherapy (ex. 5-fluorouracil) can be increased and efficacy can be reduced in hyperglycemic conditions (Garg, Maurer et al. 2014).

More controverse is the influence of anti-diabetic drugs on cancer development. Although evidence is increasing on the protective role of metformin in primary and secondary cancer prevention (see Chapter 1.5), conflicting data exists about the role of sulphonilureas, insulin and insulin analogues and the newest drugs like some GLP-1 (glucagon-like peptide - 1) based therapies as GLP-1 receptor agonists (GLP-1ras) and dipeptidyl-peptidase-4 (DPP-4) inhibitors as well as sodium-glucose co-transporter-2 (SGLT2) inhibitors.

A debate was raised in the late 00`s as a consequence of 3 independent studies conducted in Germany (Hemkens, Grouven et al. 2009), Sweden (Jonasson, Ljung et al. 2009) and Scotland (Colhoun and Group 2009) groups which pointed for an association between insulin glargine and cancer risk. The impact of these publications was so serious that the European Association for the Study of Diabetes (EASD) decided to create a special Advisory Group, which agreed that it would be premature to publish these findings in isolation, and that replication was needed. Since then, phase three studies like ORIGIN (International Outcome Reduction with Initial Glargine Intervention), a prospective 6.2 years trial whose primary endpoint was the effect of insulin glargine on CVD (Investigators, Gerstein et al. 2012) and other large database studies like the Kaiser Permanent Collaboration (KPC) were performed and failed to find any risk association (Habel, Danforth et al. 2013). The conclusion was that a multidisciplinary approach was needed to uncover the mechanisms underlying the risk associations between these diseases and, ultimately, improve clinical outcomes.

Incretin based therapies, which include GLP-1 receptor agonists and DPP-4 inhibitors, are another group of antidiabetic drugs surrounded by controversial evidence due to conflicting findings regarding the risk of acute pancreatitis for both the classes and pancreatic (exenatide) and medullary thyroid cancer (liraglutide) (Elashoff, Matveyenko et al. 2011). Pancreata from brain-dead organ donors by the Juvenile Diabetes Research Foundation (JDRF) Network for Pancreatic Organ Donors with Diabetes (nPOD) demonstrated a marked expansion of the exocrine and endocrine pancreatic compartments with incretin use (Butler, Campbell-Thompson et al. 2013). More recent studies do not seem to confirm this association. A 2018 meta-analysis from Wang et. al did not find any increased risk of pancreatic cancer with the use of incretin based therapies in T2-DM for 104 months (Wang, Liu et al. 2018). Anyway, the available data led Food and Drug Administration (FDA) and the European Medicines Agency (EMA) to conclude that there is not sufficient evidence to conclusively determine whether long term exposure to GLP-1ras increases the risk of pancreatic cancer. Longer follow-up (e.g., 10 years) was recommended to further characterize the relationship between GLP-1 receptor agonists and the development of pancreatic cancer (https://www.fda.gov/media/105560/download).

SGLT2 inhibitors are a new and promising class of drugs not only for T2-DM but also for its pleiotrophic effects. Recent studies had demonstrated a cardioprotective effect of this class of drugs (Zinman, Wanner et al. 2015, Kato, Silverman et al. 2019, Mahaffey, Jardine et al. 2019) as well as a renal protective action (Neal, Perkovic et al. 2017, Mosenzon, Wiviott et al. 2019). Their mechanism of action involves an inhibition of renal reuptake of glucose by the proximal tube, which increase glycosuria (Kalra 2014). As increased urine glucose is associated with an higher risk of bladder cancer, the question about long-term use of SGLT2 inhibitors and bladder cancer association is a major concern. Another side effect of these oral anti-diabetic agents are urinary tract infecctions that are also associated with genito-urinary cancer. A recent meta-analysis did not find an overall increased risk of cancer (Tang, Dai et al. 2017) associated with SGLT2 inhibitors. Anyway, bladder cancer risk was elevated (OR 3.87 [95% CI 1.48, 10.08]), specially with empagliflozin and a decreased gastrointestinal cancer risk (OR 0.15 [95% CI 0.04, 0.60]) was found to be

associated with canagliflozin. Nevertheless, relationship between SGLT2 inhibition and cancer formation is still inconclusive and studies with larger sample size, longer exposure duration, and different ethnicities are warranted (Lin and Tseng 2014).

However, conclusions about the pro-neoplastic influence of anti-diabetic drugs must be carefully undertaken, as T2-DM *per se* is also associated with an increase incidence of several cancers (Garg, Maurer et al. 2014).

1.4 Insulin Resistance, Inflammation, Metabolic Syndrome and Cancer

At 1988 ADA`s Banting Lecture, Reaven described for the first time the role of IR in human disease (Reaven 1988). He stated that "…resistance to insulin-stimulated glucose uptake is present in the majority of patients with IGT or non-insulin-dependent diabetes mellitus (NIDDM) and in ∼25% of non-obese individuals with normal oral glucose tolerance. In these conditions, deterioration of glucose tolerance can only be prevented if the β-cell is able to increase its insulin secretory response and maintain a state of chronic hyperinsulinemia. When this goal cannot be achieved, gross decompensation of glucose homeostasis occurs."

He also described hypertension, hyperinsulinemia, IGT, increased plasma triglyceride concentration, and decreased HDL-c, as a clustering of RFs all of which are associated with increased risk for coronary artery disease (CAD). He called this syndrome X, later renamed into Metabolic Syndrome. In his paper named "the deadly quartet", Kaplan added upper-body obesity (visceral obesity) to glucose intolerance, hypertriglyceridemia and hypertension as RFs for CVD (Kaplan 1989). Since then, this subject had a continuous development, with crescent complexity involving organs such, as the liver, the muscle, the pancreas, the cerebrum and more recently the gut.

Visceral obesity, T2-DM and CVD share a metabolic milieu characterized by IR and chronic subacute inflammation (Shoelson, Lee et al. 2006). The association

between inflammation and T2-DM were described more than one century ago when aspirin was proved to diminish glycosuria (Williamson 1901). Chronic interaction of several mechanisms lead to a state of metabolic endotoxaemia, which ultimately increases the risk of CVD throughout elevated levels of markers and mediators like fibrinogens, C-reative protein (CRP), interleukin-6 (IL-6) and tumor necrosis factor alfa (TNF-α), among others.

Tumor microenvironment alterations within the tumor are essencial for tumor metabolic reprogramming resulting in tumor progression and metastasis (Xing, Zhao et al. 2015). Tumor stromal cells, such as cancer–associated fibroblasts (CAF) and tumor associated-machrophages (TAM) are responsible for paracrine secretion of inflammatory citokines or chemokines and intermediate metabolites that activate the major intracellular signalling pathways implicated in cell proliferation, expansion, survival, adhesion, invasion and metastasis (Xing, Zhao et al. 2015, Spyrou, Avgerinos et al. 2018). Nowadays, more than 15 pro-inflammatory adypocitokines are known to be one of the connections between adiposopathy induced malignancy. The most well studied adipocitokine is leptin which has shown to promote cell proliferation, invasion, angiogenesis and inflammation in basic research studies, although these were not reproducible in human studies at physiologic conditions (Spyrou, Avgerinos et al. 2018). On the contrary, adiponectin, which has pro-insulinosensivity properties exibits anti-proliferative, anti-migratory and pro-apoptotic actions. Hypoadiponectinemia promotes tumor proliferation through the increase of anabolic hormones like insulin and IGF1 and pro-inflammatory cytokines as TNF-α and lL-6. In humans, hypoadiponectinemia has been associated with a six-fold increased risk for endometrial cancer (Petridou, Mantzoros et al. 2003) and also breast cancer incidence (Miyoshi, Funahashi et al. 2003) and invasiveness (Chen, Chung et al. 2006).

More recently it was demonstrated that obesity-related quantitative and qualitative alterations in gut microbiota with low expression of Bacteroides and high expression of Fimicutes have a fundamental role in this process (Cani, Neyrinck et al. 2007, Cani, Bibiloni et al. 2008, Cani and Delzenne 2009) by increasing permeability of enterocytes, lowering plasma lipopplysaccharide (LPS)

levels, which contribute to visceral adipose tissue inflammation, oxidative stress, macrophage infiltration, and metabolic disorders (Fig. 2).

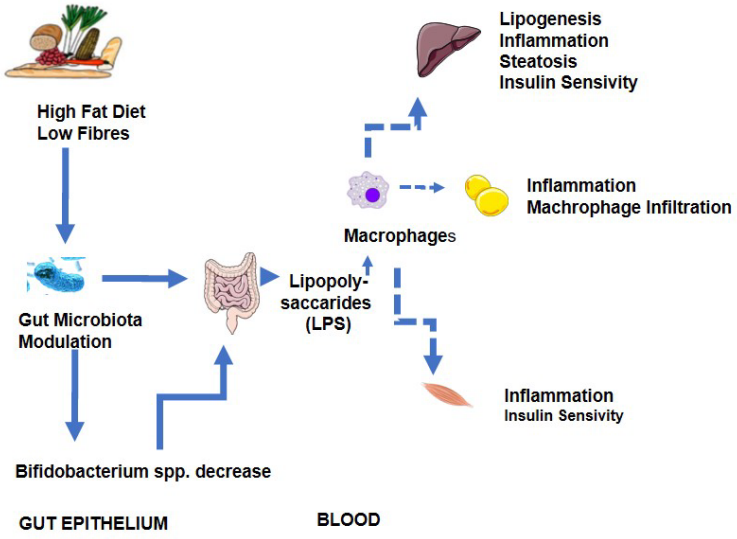

Figure 2. Schematic View of the Interaction between Diet, Microbiota, Inflammation and Insulin Resistance *(Adapted from Cani 2009)*

The gut and the pancreas are organs that secrete hormones and peptides, such as ghrelin, GLP1, peptide tyrosine tyrosine (PYY), pancreatic polypeptide (PP) and cholecystokinin (CCK), which have an important role on energy balance and maintenance of homeostasis, by inducing saciety and meal termination. They also seem to have a role on the pathgenesis of obesity (Mishra, Dubey et al. 2016).

The “Warburg hypothesis” postulated by the Nobel laureate Otto Heinrich Warburg in 1924, hypothesized that cancer is caused by the fact that tumor cells mainly generate energy by non-oxidative breakdown of glucose (glycolysis) (Fig. 3).

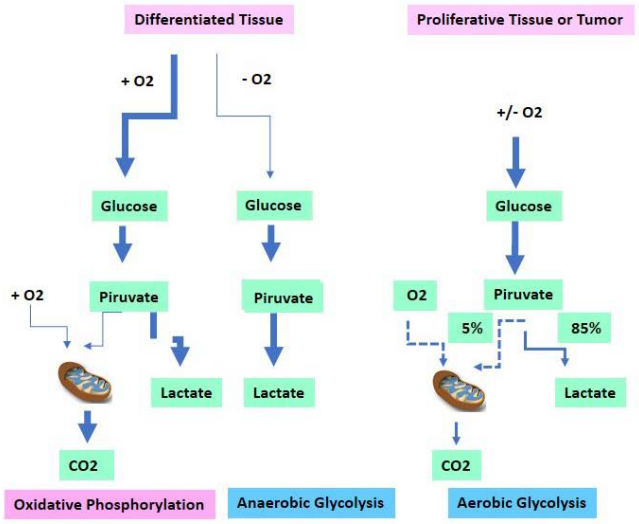

Figure 3. Schematic View of Warburg Hypothesis of Aerobic Glycolysis in Proliferating Cells *(Adapted from Brand 2010)*

According to his theory, “the prime cause of cancer is the replacement of the respiration of oxygen in normal body cells by a fermentation of sugar” (Brand 2010). Almost 50 years later, Warburg hypothesis was the basis for the development of ^{18}F-fluorodeoxyglucose Positron Emission Tomography (^{18}F-FDG-PET).^{18}F-FDG-PET transformed oncology as it is “the diagnostic machine that, in our belief, better interprets the fusion between function and form, which promotes the fractal condition” (…). PET sees the functioning organ (…). The difference on the metabolic intensity defines morphofunctional áreas” (Silva 1995). In fact, PET imaging with ^{18}F-FDG-PET has gained widespread clinical acceptance as a marker of tumor glycolysis (Pantel, Ackerman et al. 2018).

Rudolf Wirchow (Virchow 1863) was the first to hypothesize the contribution of inflammation to cancer. Chronic inflammation, regardless of origin, induces neoplastic transformation of cells and incidence of cancer can be significantly reduced by avoiding exposure to those agents or conditions that provoke tissue inflammation, such as smoking, alcohol, carcinogenic chemicals, ionizing radiation and obesity.

Several studies have demonstrated the link between inflammation and cancer: i) association between several chronic inflammatory (namely auto-immune) diseases and cancer (ex. chronic inflammatory bowel disease and cancer); ii) reduction of cancer risk with the use of anti-inflammatory agents (ex. aspirin), iii) many of the cells that are important to inflammation (ex. macrophages), are involved in cancer, iv) blocking or deleting these inflammatory molecules have anti-neoplastic effects, v) laboratory manipulation of healthy cells into cancer cells will start to produce inflammatory cytokines (Hanahan and Weinberg 2000).

Evidence between inflammation and cancer is so strong that microenvironment inflammation (Colotta, Allavena et al. 2009) has joined the previous six factors as the seventh hallmark of cancer (Table III).

Table III. The seven hallmarks of cancer

Self-sufficient growth signals
Evading apoptosis
Insentivity to anti-growth signals
Limitless replication potential
Sustained angiogenesis
Tissue invasion and metastasis
Inflammatory microenvironement

Adapted from Colotta, Allavena et al. 2009

Although there is solid epidemiologic evidence on the association of obesity and cancer, the interest in investigating the molecular mechanisms underlying obesity-related cancer is more recent. Inflammatory mechanisms that promote tumorigenesis (Font-Burgada, Sun et al. 2016) involve hormones, adipokines, cytokines and immune infiltration that characterizes the subclinical systemic inflammation associated with visceral adipose tissue hyperplasia and hypertrophia. Thus, obesity is often denominated “ the oil that fleeds the flame” (Font-Burgada, Sun et al. 2016).

Kaaks authored an historical publication that was one of the first to describe insulin as the missing link between obesity and breast cancer risk (Kaaks 1996). Since then, that field was subject of a great development and nowadays the mechanisms responsible for the association between IR and cancer are becoming more clear. Insulin is an anabolic hormone with direct and indirect pro-proliferative effects via IGF1 pathway stimulation. Genetic susceptibility and environmental factors in association with visceral adipose tissue, IR, hyperinsulinemia, elevated levels of insulin-growth factors, hyperglycemia, increased free fatty acids (FFA) and TG, lead to a milieu that favours cell proliferation, DNA damage, anti-apoptosis, migration and angiogenesis which ultimately cause neoplastic transformation of cells. Depressed auto-immunity and high hormone levels, such as estrogens in hormone-dependant breast cancers, or testosterone androgen-dependent prostate cancer also contribute to cell proliferation (Jee, Kim et al. 2005) (Fig. 4).

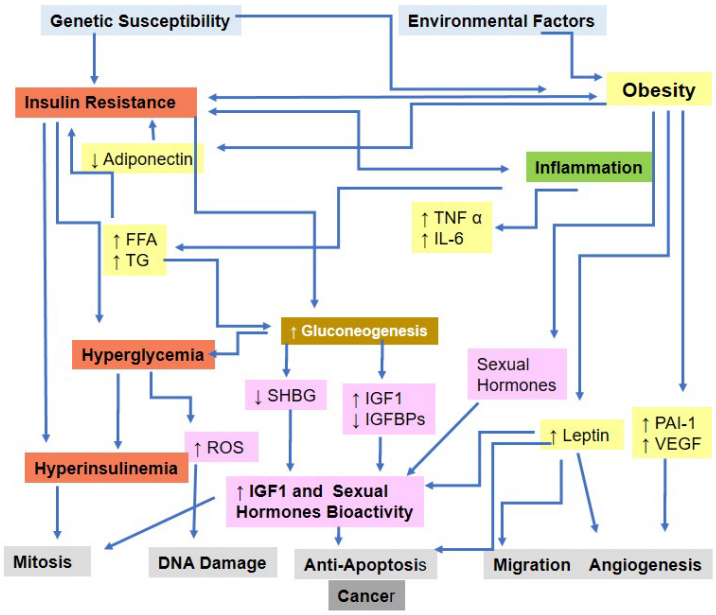

Figure 4. Schematic View of the Proposed Model for Insulin Resistance Link with Increased Cancer Risk *(Adapted from Jee 2005)*

Another emerging factor is the contribution of microbiota for cancer risk. As a matter of fact, altered microbiota has several effects on metabolism, cellular proliferation, inflammation and immunity, besides influencing several other cancer predisposing conditions, initiation, genetic instability, susceptibility to host immune response, progression, comorbidity and response to therapy (Roy and Trinchieri 2017).

Changes in intestinal microbiota and development of digestive cancers as pancreatic adenocarcinoma and colorectal cancer is now established. Functional studies in animal models have pinpointed the role of several bacteria, including Fusobacterium nucleatum and certain strains of Escherichia coli and Bacteroides fragilis in colorectal carcinogenesis (Wong SH, 2019). Also, molecular studies revealed that dysbiosis of microbiota, namely biliary microbiota, also influences pancreatic cancer development (Karpinski 2019). Recent focus has been made on the modulation of cancer immunotherapy efficacy by gut microbiota and the role of alteration by faecal transplantation in order to improve immunotherapy response (Wang, Ma et al. 2018, Huo, Liu et al. 2019).

In conclusion, obesity, IR, CVD and cancer share many underlying mechanisms that support the hypothesis that cancer should be considered a co-morbidity of obesity and T2-DM.

1.5 Implications for Cancer Prevention and Treatment

Overweight and obesity constitute a global pandemic with devastating consequences that affect 2 billion people. Obesity plays a central role in morbidity and mortality of diseases of multiple organs and systems, and it is a major contributor to the growing incidence of cancer. There is now enough level of evidence for the association between overweight and 13 types of cancer, among which are two of the most common cancers worldwide, those of the colon-rectum and postmenopausal breast. Sedentary lifestyle, unhealthy diet, and excessive alcohol intake also account for the burden of cancer by promoting obesity. The risk of specific types of cancer is also directly influenced, regardless of the

magnitude of adiposity, by physical inactivity, consumption of red meat, processed meat and ultra-processed foods, dairy products, alcohol, and low consumption of whole grain cereals, nuts, vegetables, and fruits.

T2-DM is another global health threat closely associated with obesity that boosts the risk of cancer driven by high BMI (Lopez-Suarez 2019). Since 1980, childhood overweight and obesity prevalence has doubled in more than 70 countries worldwide. Adolescent obesity is associated with increased morbidity and mortality in midlife due to CV or metabolic disorders (Berenson and Bogalusa Heart Study 2012) and there is also increasing level of evidence that childhood obesity is associated with increased cancer risk in adulthood (Lauby-Secretan, Scoccianti et al. 2016, Shamriz, Leiba et al. 2017).

Nurses´ Health Study (NHS), the Health Professional Follow-up Study (HPFS), and the National Health and Nutrition Examination Survey (NHANES) 2013–2014 had constructed a score with 5 lifestyle RFs including smoking, BMI, alcohol intake, physical activity, and a high diet quality score (upper 40%). Data revealed that the population-attributable risk of nonadherence to the five RFs was 60.7% for all-cause mortality, 51.7% for cancer mortality, and 71.7% for CVD mortality. In women and men at the optimum level of the five RFs, the projected life expectancy at age 50 years was 14.0 and 12.2 years longer, respectively when compared with those at the worst level (Li, Pan et al. 2018).

Although the future projections of the increase incidence of obesity and diabetes epidemics are scaring, with an enormous impact on and cancer morbidity and mortality, the finding that most of the cancers are attributable to modifiable RFs opens a field of hope.

The impact of nutrition on cancer primary prevention as well as in the prognosis, led several organizations like the WCRF/AICR to state several recommendations on healthy alimentary habits with preference for a mediterrranean type-diet (www.dietandcancerreport.com).

Physical exercise (PE) is the second triangle side of the triad of factors that can prevent obesity-related cancers. Everybody has heard about the walkings and runnings against cancer organized by several patient and medical organizations.

In fact, a scientific explanation exists, as PE is beneficial not only for primary prevention, but also has impact on outcomes of pre-diagnosed individuals (Lugo, Pulido et al. 2019). Postulated mechanisms include decreased levels of reactive oxygen species (ROS), enhancement of immune function, decreased levels of inflamation, and improved insulinsensitivity (Marzatico, Pansarasa et al. 1997, Bradley, Jeon et al. 2008, Pedersen, Idorn et al. 2016). Moderate physical activity (PA) also induces gene expression of anti-oxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, which protect against oxidative DNA damage (Selamoglu, Turgay et al. 2000, Fisher-Wellman, Bell et al. 2009). Furthermore, PA changes the metabolic profile of estrogens, leading to reduced hormonal activity and increased anti-proliferative properties in breast cancer patients (Zhu and Conney 1998). Table IV shows PE established benefits on specific cancers

Table IV. Effects of Physical Activity on Malignancy

Malignancy	Effects of Physical Activity
Colorectal	• Reduces incidence of colorectal cancer among men and women. • Decreases mortality of colorectal cancer when performed before or after a diagnosis. • Reduces fatigue among patients receiving chemotherapy and improves quality of life
Breast	• Decreases risk of breast cancer. • Any level of physical activity before or after the diagnosis significantly decreases the relative risk of total and breast cancer-specific mortality. • There appears to be a linear dose response curve between volume of physical activity and cancer recurrence.
Prostate	• Mixed findings regarding the relationship between physical activity and incidence of prostate cancer. • Few studies have observed a higher incidence of prostate cancer among patients with lower levels of physical activity. • Post-diagnosis physical activity was associated with lower prostate cancer-specific mortality as well as better mental and physical quality of life.
Lung	• Higher levels of physical activity appear to reduce risk. • Preoperative physical activity may confer benefits to patients undergoing lung cancer surgery.
Endometrial	• May decrease endometrial cancer risk. • The benefits of physical activity on survival after endometrial cancer are unknown.

Adapted From Lugo D, 2019

Both healthy lifestyle interventions that improve insulin sensivity and reduce hyperinsulinism could reduce cancer risk in almost 30% (Grundy, Poirier et al. 2017).

On the third side of the triangle, chemoprevention is a promising field and object of intensive investigation.

Metformin, the most well studied drug at that level, as showed unquestioned benefits not only in vitro and animal studies (Rizos and Elisaf 2013), but also epidemiological and prospective trials in humans (Zi, Zi et al. 2018). Evidence about in vitro and in vivo anti-cancer effects of metformin began at early 00`s, and gained focus on the 10`s, being well described in two provocative papers intituled “Metformin – taking the candy for cancer” (Jalving, Gietema et al. 2010) from Netherlands and “Metformin: a diabetes drug for cancer or a cancer drug for diabetes” (Martin and Marais 2012) from UK.

A recent review (Zi, Zi et al. 2018) addresses not only the epidemiological evidence about the antitumor effects of metformin, but also as the mechanisms through which metformin exercises its anticancer effect, the synergism with other drugs and also its influence on cancer stem cells.

The anti-cancer mechanisms of action of metformin are mainly indirect through improvement of IR and decreasing hyperinsulinism by inhibition of the insulin-IGF1 axis, and direct effects through the AMPK/PI3K/Akt/mTor pathway (Fig. 5).

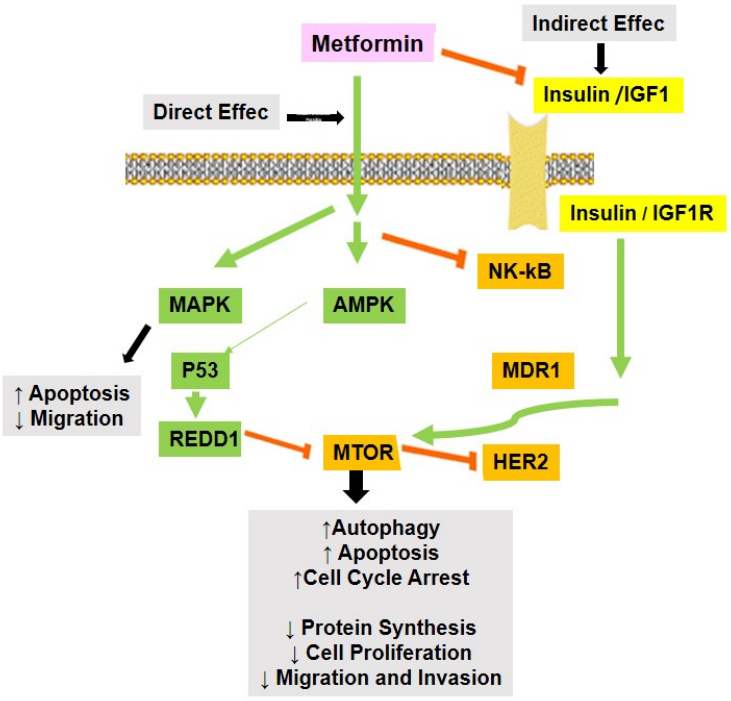

Figure 5. Schematic View of Anti-Cancer Mechanisms of Metformin *(Adapted from Lei 2017)*

It has also immune-mediated anti-cancer effects by enhancing CD8+ T cells which are the key players in mediating immunity to tumors (Lei, Yi et al. 2017, Yu, Mao et al. 2017).

Interestingly, recent discoveries about the glucose lowering effects of metformin show that intestine is another important site of the action of this drug by activation of intestinal mucosa AMPK that could stimulate the release of GLP-1 and PYY (Buse, DeFronzo et al. 2016, Foretz, Guigas et al. 2019). Moreover, lower intestine is responsible for at least of 70% of the maximal glucose-lowering effect of metformin.

Although not so extensively studied as metformin, other therapies used in the individual components of metabolic syndrome are also being tested in cancer.

There is still a great controversy on the anti-neoplastic effect of statins. Preclinical studies suggest an anti-tumor action on several types of cancer cells throughout inhibition of tumor cell growth, inhibition of angiogenesis, induction of apoptosis and repression of tumor metastasis, by intervening on mevalonate pathway and

thus inhibiting Ras-MAPK and PI3K/Akt pathways, as well as the induction of apoptosis through the regulation of anti- and pro-apoptotic proteins in cancer cells (Hindler, Cleeland et al. 2006). In clinical studies evidence is not unanimous. A recent metanalysis from Italy described the actual evidence of different statins in reducing the incidence of cancer and mortality related to cancer in general (Iannelli, Lombardi et al. 2018). On the other side, a metanalysis from Canada of the same year (Farooqi, Malhotra et al. 2018) did not show any benefit on OS and progression free survival (PFS) of adding statins to conventional anti-cancer therapy on advanced tumors, with a prognosis equal or less than two years.

The effect of metabolic surgery in the incidence and prognosis of cancer has also been studied, although there are few publications about this matter. One of the first was reports comes from the prospective Swedish Obesity Study (SOS) (Sjostrom, Gummesson et al. 2009). Subjects submitted to bariatric surgery who lost more than 20 kg and were followed for more than 10 years, had a significant reduction of cancer risk, specially women (RR 0·58, 95%CI 0·44–0·77) rather than men (RR 0·97, 0·62–1·52). More recently, Schauer et. al. (Schauer, Feigelson et al. 2017) showed that the percentage of weight loss at one year after bariatric surgery in 18 355 subjects, was significantly associated with a reduced risk of any cancer in adjusted models (HR 0.897, 95% CI 0.832–0.968, p=0.005 for every 10% weight loss).

The role of peroxisome proliferator-activated receptors (PPARs) as anti-cancer agents has also been studied. PPARs are a group of nuclear receptors (NRs) that play essential roles in the regulation of several physiological processes, including cellular differentiation and development, whole-body energy homeostasis (carbohydrate, lipid, protein) and tumorigenesis (Hong, Xu et al. 2018). Among the available drugs that target PPAR, the most used in clinical practice are fibrates for hypertriglyceridemia and low HDL-c, characteristics of MetS (acting on PPAR α) and pioglitazone, an insulin sensitizer used for T2-DM treatment (acting on PPAR γ). There is still a great controversy whether drugs targeting PPARs are friend or foe in cancer treatment. Although cell proliferative stimulation by PPAR α receptor activation is controversial, PPAR γ agonists seem to have anti-cancer properties, namely in non-small cells lung cancer (NSCLC)

(Pishvaian, Marshall et al. 2012) and chronic myeloid leukaemia resistant to imatinib (Yousefi, Shafiei-Irannejad et al. 2016).

The potential role of anti-hypertensive drugs in cancer treatment is the less studied among the drug classes used to target MetS syndrome features. A 2015 review on the potential anti-cancer properties of drugs commonly used for other indications (Hanusova, Skalova et al. 2015), points out the anti-cancer effects of α and β blockers through cytostatic properties, namely the pro apoptotic effects of the former. Some studies point also to the oncolytic action of ACE inhibitors by reducing vascular endothelial growth factor (VEGF) intra-tumoral levels (Radin, Krebs et al. 2018). Long-term use of angiotensin II type receptor antagonists (ARA II) seems to act on different cancers (lung metastasis of renal cancer and prostate cancer) also by anti-angiogenesis properties (Tadic, Cuspidi et al. 2019). A recent work from Basel (Benjamin, Robay et al. 2018) described the dual effect of metformin and the anti-hypertensive syrosingopine by blocking the pathways that generate Nicotinamide adenine dinucleotide+ (NAD+) from Nicotinamide Adenosine Dinucleotide Hydride (NADH), depleting energy supply and dead of cancer cells.

Summarizing data above, the burden of cancer is increasing worldwide. Cancer awareness, early detection as well as better access and improvements in treatment, led to a small decrease in mortality in Europe (-1.3% in 6 years) (Ferlay, Colombet et al. 2018). However, decline in cancer mortality from mid-1990`s to 2010 was only 10%, compared with the 35% CVD mortality decrease between 2002-2012 (Malvezzi, Carioli et al. 2018). The fact that one third to one half of the cancers can be prevented is of major importance and led to the development of the European Code Against Cancer (Anderson, Key et al. 2015), a project from the WHO and the IARC co-funded by the European Community, with 12 recommendations to reduce cancer risk (Table V).

Table V. European Code Against Cancer (12 Ways To Reduce Your Cancer Risk)

- Do not smoke. Do not use any form of tobacco
- Make your home smoke-free. Support some-free activities in your workplace
- Take action to be a healthy good weight
- Be physically active in everyday life. Limit the time you spend sitting
- Have a healthy diet: eat plenty of whole grains, pulses, vegetables and fruits; limit high calorie foods (foods high in sugar or fat) and avoid sugary drinks; avoid processed meat, limit red meat and foods high in salt
- If you drink alcohol of any type, limit your intake. Not drinking alcohol is better for cancer prevention.
- Avoid too much sun, especially for children. Use sun protection. Do not use sunbeds
- In the workplace, protect yourself against cancer-causing substances by following health and safety instructions
- Find out if you are exposed to radiation from naturally high radon levels in your home. Take action to reduce high radon levels
- For women: breastfeeding reduces the mother`s cancer risk. If you can, breastfeed your baby. Hormone replacement therapy (HRT) increases the risk of certain cancers. Limit use of HRT.
- Ensure your children take part in vaccination programmes for Hepatitis B (for new-borns) and Human papillomavirus (HPV) for girls
- Take part of organized cancer screening programmes for: bowel cancer (men and women); breast cancer (women) and cervical cancer (women)

Adapted from Anderson, Key et al. 2015

European Code Against Cancer stresses for the major importance of maintaining a normal body weight, adopting a healthy diet and promoting physical exercise on cancer prevention becomes evident. These lifestyle recommendations can also be applied to cancer patients for secondary prevention and have been demonstrated to influence cancer mortality. In a near future, chemoprevention of cancer using drugs that act on metabolic factors that lead to cancer development is also promising.

This modern way of cancer aproach is a subject of concern of European authorities and led to the creation of the consortia Cancer Prevention Europe (CPE) in 2018 (Wild, Espina et al. 2019). The idea of increase international research collaborations and improve interaction between the different components needed for a coherent cancer prevention research continuum, derived from the importance of connecting the expanding knowledge in basic

cancer biology and preclinical research, which requires a critical mass of expertise and resources, as well as large number of patients (Ringborg 2019).

European authorities created a mission called Horizon Europe (Celis and Heitor 2019). The mission states that “by combining innovative prevention and treatment strategies in a sustainable state-of-the-art virtual European cancer centre/infrastructure, it will be possible by 2030 to achieve long-term survival of three out of four cancer patients in countries with well-developed healthcare systems”. In the long-term, primary prevention will change the increasing cancer incidence. Recent estimates of preventable fractions of cancer suggest that about half of all cancer cases could be prevented through rigorous implementation of successful prevention measures, among other actions, by following the cancer prevention recommendations of the European Code against Cancer (Schuz, Espina et al. 2019). This project will envolve a consortia of European Compreensive Cancers Centers (ECCC), the actual core constituted by seven centers. Portugal cannot be out of the project as several Portuguese authorities were part of the main boosters of the creation of the project Cancer Core Europe (CCE) in 2014 (Eggermont, Apolone et al. 2019).

1.6 Gastro-Entero-Pancreatic Neuroendocrine Neoplasia

1.6.1 Gastro-Entero-Pancreatic Neuroendocrine Neoplasia Burden – another recent epidemic

NEN are an heterogeneous group of rare malignancies originating from endodermal cells with secretory capacity within the neuroendocrine system. GEP-NEN represent a subtype of these tumors, located either in the pancreas or in the gastrointestinal tract. Although GEP-NEN incidence is still relatively low, the age adjusted incidence rate increased 6.4-fold from 1973 (1.09 per 100,000 persons) to 2012 (6.98 per 100,000 persons) (Dasari, Shen et al. 2017). Recent statistics show that although the tendency of non-neuroendocrine tumors incidence was to towards stablity or even to slightly decrease in developed

countries, the trend on neuroendocrine tumors incidence is still rising (Dasari, Shen et al. 2017). Localized disease with special focus on incidentally detected tumors by endoscopic techniques gastric and rectal NEN are the tumors that most contribute for the statistics. Due to the long survival rate of patients, the estimated 20-year limited-duration prevalence of NEN in USA on January 1, 2014, was 171,321 (Yao, Hassan et al. 2008), twice than the 100 000 registered in 2008. The increase in GEP-NEN prevalence could also be attributed to the decrease in mortality rate from 17.1% in 2005-2008 to 21.3% in 2009-2012, derived from the improvements in diagnostic methods, precise classification of the disease, as well as implementation of early and more effective treatments. Nevertheless, epidemiological trend analysis using national statistics from several countries suggest that in order to explain the difference in geographic and ethnic incidence patterns, both genetic and environmental factors must be involved in the natural history of NEN. The actual mechanisms leading to this recent burden have not attracted extensive investigation and remain largely unknown (Huguet, Grossman et al. 2017).

So, although GEP-NEN are rare, recent burden of disease may suggest that in a lower scale we are also facing a new epidemic.

1.6.2 Gastro-Entero-Pancreatic Neuroendocrine Neoplasia Studies on Risk Factors

NEN can occur at any age, but most cases arise in people with median age of 59 y (10-102 y) (Borbath, Pape et al. 2018). The number of new cases of NEN diagnosed each year has been rising for about the last 40 years. The most recent data from SEER (Surveillance, Epidemiology, and End Results) Program revealed that annual incidence increased nearly seven-fold with a current prevalence of 6.98 cases/100,000 inhabitants (Dasari, Shen et al. 2017). The general idea from researchers is that this incidence rise is due to better diagnostic tools, especially imaging tests and improved diagnostic skills from medical community. Anyway, there are very scarce data about the cause of these rare tumors, as well as about the etiopathogenesis of the disease.

A search on Pubmed® on 8th December 2019 with the keywords “neuroendocrine tumors treatment” showed 92360 references. But when the keywords changed into “neuroendocrine tumors risk factors”, 7245 references were showed, most of them related to variables linked to the clinical presentation or tumor`s characteristics, which include tumor and metastasis molecular RFs. The number of studies is even lower if keywords were “neuroendocrine tumors clinical risk factors” (2482 references) and again most of them related to the tumor itself and not due to external variables.

The only convincing evidence on RFs for NEN so far identified are hereditarity and familial history of non-endocrine cancer (Hassan, Phan et al. 2008). Hereditarity is responsible for only 5-10% of GEP-NEN (Anlauf, Garbrecht et al. 2007), most of them included in MEN1 syndrome, being either pancreatic NEN (panNEN) or broncho-pulmonary NEN (BP-NEN). Von Hippel-Lindau syndrome, Neurofibromatosis type 1 and Tuberous Sclerosis Complex are also hereditary syndromes with a higher risk of developing panNEN (Lodish and Stratakis 2010).

In case of non-familial cancer, first relatives with any type of non-neuroendocrine cancer face an increased risk for NEN as well. A italian cohort found an increased risk of NEN of the small intestine NEN (SI-NEN) in case of family history of colorectal or breast cancer (Rinzivillo, Capurso et al. 2016). Whether this is related to a genetic association or exposure to enviromental factors among family members is still unknown. Other possible RFs are smoking (Rinzivillo, Capurso et al. 2016), and diabetes, while data on whether obesity, dietary patterns and sedentary lifestyle are RFs as well, is either scarce or conflicting. RFs for panNEN are also conflicting, as some RF described in older studies like smoking, alcohol and first-degree family history of cancer (Capurso, Falconi et al. 2009) were not confirmed in a recent publication from the same group (Valente, Hayes et al. 2017). The only factor that was maintained was the occurrence of previous diabetes (Valente, Hayes et al. 2017).

The evidence that supports that obesity and diabetes could be a RF for neuroendocrine neoplasia is scarce and solely based on case-controlled studies performed mainly in non-functioning panNEN.

A metanalysis performed by Leoncini et. al. (Leoncini, Carioli et al. 2016) concluded that obesity confers an estimate OR of 1,37 (95%CI 0.25-7.69, I^2=98.5, p<0.0001) of developing panNEN, although this conclusion is not supported by some of the studies. Concerning T2-DM, the available data is more homogeneous to suggest that diabetes confers an OR 2.76 risk of developing NEN (95%CI 1.65-4.64, I^2 =55.3, p=0.090), the risk is even higher for recent onset diabetes (OR 12.80, 95%CI 2.47-66-42, I^2 = 55.3%, p=135). More recently, in vitro experiments demonstrated a reduction of the expression of somatostatin receptor (SSTR) subtypes in lung and GEP-NEN cells of patients with diabetes versus non diabetics (Herrera-Martinez, Pedraza-Arevalo et al. 2019).

The link between meuroendocrine neoplasia and metabolic abnormalities that include hyperglycemia and dyslipidemia is also reflected on the side effects of the majority of approved systemic therapies. Hyperglycemia is a frequent side effect in patients under SA, tirosine kinase inhibitors (TKIs) and also mTor inhibitors by an IR increase mechanism through mammalian target of rapamycin complex 1 (mTORC 1) activity (Verges, Walter et al. 2014). As a consequence they also promote an elevation of TC, LDL-c and TG (Verges, Walter et al. 2014). Interestingly, mTor inhibitors seem to increase HDL-c, the mechanisms involved in this increment are still under investigation.

1.6.3 Inflammation and Metabolic of Aspects Neuroendocrine Neoplasia

NEN are subject of intensive investigation in the last 20 years. The 2000 (Solcia, Klöppel et al. 2000) and posterior 2010 WHO classification of NENs according to proliferation grade (Bosman, Carneiro et al. 2010) was a great advance for establishing guidelines on management and treatment (https://nanets.net/net-guidelines-library,www.esmo.org/Guidelines/Endocrine-and-Neuroendocrine-Cancers; https://www.enets.org/enets_guidelines.html).

In 2017 WHO classification was revised again (santé and cancer 2017) focusing also in tumor morphology and subdividing neuroendocrine carcinoma into 2 classes for panNEN (Table VI).

Table VI. 2010 and 2017 WHO Classification of Neuroendocrine Neoplasia

WHO 2010*		**WHO 2017**(for pancreatic neoplasms)**	
Well Differentiated Neuroendocrine Tumors		**Well Differentiated Neuroendocrine Tumors**	
NETG1 (neuroendocrine tumors grade 1)	< 2 mitosis/ 10 HPF ≤ 2% Ki67 index	NET G1	<2 mitosis/ 10 HF <3% Ki67 index
NETG2 (neuroendocrine tumor grade 2)	2-20 mitosis/ 10 HPF 3-20% Ki67 index	NET G2	2-20 mitosis/ 10 HPF 3-20% Ki67
-	-	NET G3	> 20 / 10 HPF > 20 % Ki67 index
Poor Differentiated Neuroendocrine Carcinoma		**Poor Differentiated Neuroendocrine Carcinoma**	
Neuroendocrine carcinoma	>20 / HPF > 20 % Ki67 index	Neuroendocrine carcinoma Small cell type Large cell type	>20 / 10 HPF > 20% Ki67 index
Mixed endocrine-exocrine cell neoplasm		Mixed neuroendocrine-nonneuroendocrine Carcinoma (MiNEN)	

*Adapted from *Bosman, Carneiro et al. 2010 **santé and cancer 2017*

Several prognostic markers have been characterized, which are mostly intrinsic factors to the primary tumor (PT) and to tumor metastasis (Oberg, Modlin et al. 2015). Besides established serum, tissue markers and imaging features, there is also evidence that incretin hormone receptors, mainly glucose-dependent insulinotropic polypeptide (GIP) receptors that are widely distributed in pancreatic, midgut and lung NENs independently of being functioning or non-functionig tumors, which is not observed in normal tissues or other cancers (Waser, Rehmann et al. 2012).

GLP1-receptor has been found to be present in 90% of insulinomas and exendin-3 has been used as a new agent for ^{68}Ga-DOTA-exendin-3-PET for detection of insulinomas which generally lack somatostatin receptor subtype 2 and are not detected with conventional somatostatin analogues scintigraphy (Brom, Oyen et al. 2010).

NEN`s association with inflammatory bowel disease, such as ulcerative colitis (Nascimbeni, Villanacci et al. 2005) and Chron`s disease (Boltin, Levi et al. 2011) support the hypothesis of an inflammatory basis for neuroendocrine cell neoplastic transformation. Markers of chronic inflammation and elevated pro-inflammatory cytokines were found in NEN. Berkovic beautifully described the importance of the elevation of pro-inflammatory citokines as interleukin-2 (IL-2) in functioning and interleukin-6 (IL-6) in non-functioning panNEN and GI-NEN (Berkovic, Cacev et al. 2014).

TAM was also shown to have a role in tumor iniciation and progression in several studies (Cai, Michelakos et al. 2019), traduced in a poorer prognosis and progression in several malignancies as breast, prostate and thyroid cancer and also in Hodgkin`s lymphoma (HL) (Poh and Ernst 2018). Wei et. al. described for the first time the role of TAM as an useful biomarker to predict recurrence after surgical resection of non-functional panNEN (Wei, Harmon et al. 2014).

FOXM1 is a crucial transcription factor in neoplastic cells and has been associated with differentiation and proliferation. Briest et. al. found that FOXM1 is strongly associated with tumor undifferentiation and occurrence of metastases in GI-NEN (Briest, Berg et al. 2015). In vitro studies have found that FOXM1 is associated with Ki67 index and mitotic proteins involved in NEN biology and are inhibited by siomycin A, a proteasome inhibitor which target FOXM1, thus concluding that FOXM1 is a clinical prognostic factor and a therapeutic target for GEP-NEN.

As previously mentioned, peripheral IR stimulates the pancreas to hypersecrete insulin in an attempt to maintain glucose homeostasis, inducing a state of hyperinsulinism, which is observed years before T2-DM diagnosis. Metabolic effects of insulin are mediated by the activation of Pi3K/Akt/mTor pathway. Insulin is a mitogenic hormone that acts on target cells by direct and indirect mechanisms. Insulin binds and activates the related IGF1R that is homologous to insulin receptor, but much more potent in terms of mitogenicity and transforming activity (Vigneri, 2009). Besides, insulin decreases IGF1 and 2 binding proteins, thus increasing the levels of free IGF1, the bioactive form of the growth factor. Insulin receptor expression is also increased in cancer cells, for

example in breast cancer (Papa, Pezzino et al. 1990). Another interesting aspect of the mitogenic action of insulin, is that in the presence of hyperinsulinemia, a paradoxicall shift from metabolic effects to the mitogenic effect is observed (Fig. 6).

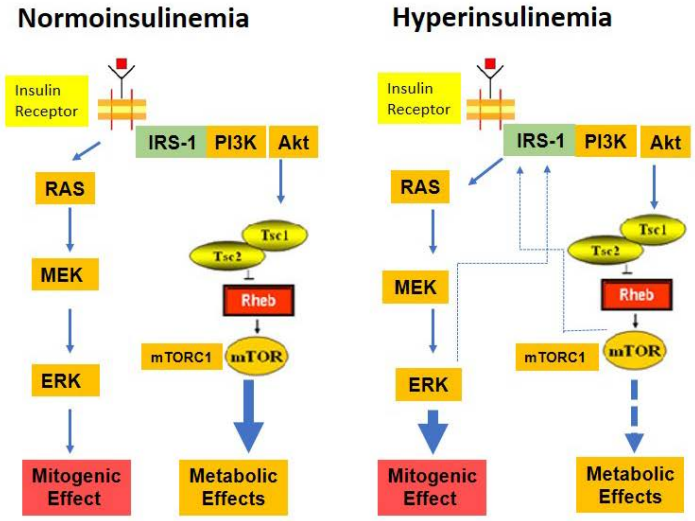

Figure 6. Schematic View of Insulin Resistance Paradox *(Adapted from Vigneri 2009)*

In the presence of IR, mTor overactivation results in the impairment of IRS-1 phosphorylation, which represents a negative feedback loop for attenuating metabolic activity in response to hyperinsulinemia. In turn, extracellular-signal-regulated kinase (ERK) pathway is activated (Vigneri, Frasca et al. 2009). As a consequence, hyperinsulinemia impairs glucose homeostasis in typical insulin targets such as liver, adipose tissue, and muscle, while exerting a cell proliferative effect on tissues that are not usual insulin targets, like ovary, breast and colon, which is in the origin of the “ectopic” proliferative activity.

Malignant transformation, tumor progression and dissemination of neuroendocrine cells involve several signalling cascades that include receptor tyrosine kinases (RTKs) and GPCRs (G-protein coupled receptors) downstream signalling, which regulate Ras/Raf, MAPK, PI3K-Akt-mTOR and JNK (c-Jun N-terminal kinases), ultimately leading to DNA synthesis and cell proliferation. NEN

are high vascularized and pro-angiogenic factors such as VEGF, platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF), transforming growth factor (TGF-α and -β) and placental growth factor (PIGF) are expressed both in panNEN and GI-NEN (Briest F, 2014).

So, PI3K-Akt-mTor signalling is one of the key molecular pathways linking metabolism and neoplasia, namely in NEN. Actually, inhibition of mTor pathway by everolimus was the basis for the approval for using this mTor inhibitor in the treatment of advanced, irressectable WD panNEN (Yao, Shah et al. 2011, Yao, Fazio et al. 2016). The major problem of these target-specific inhibitors is that the complexity and crosstalk of the different pathways involved, can result in escape phenomena that limits their clinical use (Briest F, 2014).

1.6.4 Endocrine Feedback Mechanisms in Neuroendocrine Neoplasia

Gastric neuroendocrine neoplasia (GEN) is a group of neuroendocrine digestive neoplasia. The actual classification of GEN considers four groups with different pathophysiology and clinical behaviour. Among them, type 1 GEN (T1-GEN) pathophysiology is the best understood. T1-GEN arise from enterochromaffin-like cells (ECL) and were formerly denominated ECLomas. These tumors represent an excellent example of the interaction between immune and endocrine systems, besides contributing to support the role of endocrine feedback mechanisms in the development of neoplasia.

Observing Fig. 7 we can understand that anti-parietal cell antibodies (APCAs) that typically characterize auto-immune chronic gastritis, destroy parietal cells diminishing gastric acid output at the stomach corpus (1) Gastric mucosa atrophies (2). High pH gastric content stimulates G cells to secrete increased amounts of gastrin (3). Gastrin reaches corpus gastric glands and causes ECL-cell hyperplasia (4), which can then lead to T1-GEN (Grozinsky-Glasberg, Alexandraki et al. 2018).

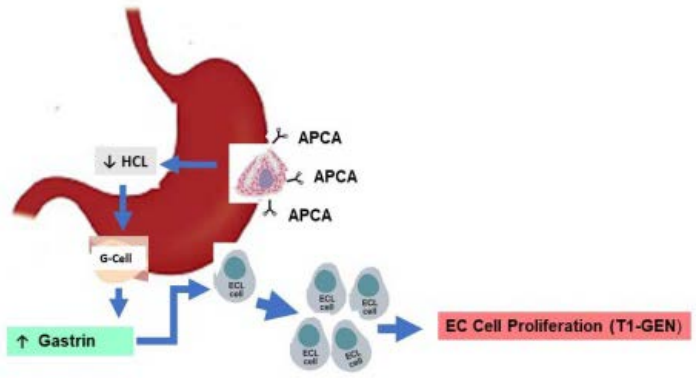

Figure 7. Schematic View of Endocrine Feed-Back Mechanisms in Type 1 Gastric Endocrine Neoplasia *(Adapted from Grozinsky-Glasberg, 2018)*

So, although the mechanisms that lead to neuroendocrine cell proliferation and NEN development in general remain largely unknown, for the particular case of T1-GEN, an endocrine positive feedback mechanism was clearly demonstrated. As a chronic inflammatory condition, T1-GEN auto-immune gastritis lead to the lack of some substance, in this case gastric acid, that through an endocrine positive feedback mechanism, stimulates the production of another substance, in that case gastrin, as an attempt to compensate for the low gastric acid output in order to restore homeostasis. On the other hand, gastrin has deleterious effects on corpus gastric glands causing cell proliferation and tumor development. So, the intrinsic connection between immune and endocrine system and neoplasia also could be applied to T1-GEN. In addition, there are some publications linking this group of neuroendocrine neoplasia to obesity and diabetes (Mottin, Cruz et al. 2009, Al-Harbi, Shakir et al. 2013). Furthermore, antral G-cell hyperplasia was observed in obese Zuker rats when compared to lean animals and dietary restriction in these animals was demonstrated to reduce antral G-cell hyperplasia to a similar magnitude as observed in lean animals (Campos, Pederson et al. 1990).

Chapter 2 - Hypothesis and Aims: Inflammation, Metabolic Syndrome and Neuroendocrine Neoplasia

"…In spite of this, oncology maintains that tumor is the primary factor in cancer, and systemic effects are secondary. But what if it is the other way around and cancer is first of all a cachexia accompanied by the tumor? At least this would explain why in most cancers' treatment fails. Take for instance arteriosclerosis that is manifested by local phenomena, e.g. stroke and myocardial infarction, and yet is essentially systemic. The same could apply to cancer which, like arteriosclerosis, is 'metabolically' systemic, and presents itself also by local phenomena, e.g. tumor. In the same way as treatment of an ailing heart does not cure the underlying arteriosclerosis, tumor removal does not cure cancer."

….in New Cancer Hypothesis G. ZAJICEK (Zajicek 1996)

Neuroendocrine Neoplasia of the Gastro-Entero-Pancreatic system has suffered an exponencial rise in the last 40 years. In an attempt to understand this recent burden, countries are making a great effort to have national registries in order to know better their reality concerning these tumors. Although the Portuguese medical community is aggregating efforts on this field, the reality of GEP NEN in Portugal is largely still unknown. Evidence on the influence of obesity, MetS and T2-DM on non-neuroendocrine cancer burden and progression is arising. The role of a chronic subclinical inflammation state associated with IR and hyperinsulinism as one of the pathogenic mechanisms that lead to neoplasic transformation of cells is also stated. The literature concerning the association of obesity, MetS and T2-DM and NEN is scarse and controverse.

To test this hypothesis, the following aims have been established:

- To perform an observational study to have an outline of GEP-NEN patients followed at the main Portuguese hospitals regarding their socio-demographic and clinic profile – a study performed in the context of the GE-TNE of SPEDM.

- To evaluate the possible association between MetS and MetS individual components with WD GEP-NEN by performing a case-control study comparing patients data from a large tertiary cancer center with a matched control group derived from the background general population, the PORMETS Study.

- To evaluate whether the presence of MetS and individual MetS components at the time of WD GEP-NEN diagnosis was associated with any specific tumor characteristics, as NEN primary site, hormone secretion, WHO grading and European Neuroendocrine Tumors Society (ENETS)`s TNM classification system, that were likely to influence the tumor biological behavior and disease prognosis.

- To identify putative molecular signatures linking WD GEP NEN and MetS to gain further insight into potential mechanisms for the association between MetS and MetS individual components with WD GEP-NEN.

Chapter 3 - Publications

3.1 Gastro-Entero-Pancreatic Neuroendocrine Neoplasia Characterization in Portugal: Results from the NETs Study Group of the Portuguese Society of Endocrinology, Diabetes and Metabolism

3.1.1 Abstract

Background: The incidence of GEP-NEN has been increasing in the last five decades, but there is no large-scale data regarding these tumors in Portugal. We conducted a cross-sectional, multicentric study in main Portuguese centers to evaluate the clinical, pathological and therapeutic profile of GEP-NEN.

Methods: From November 2012 to July 2014 data from 293 patients diagnosed with GEP-NEN from 15 centers in Portugal was collected and registered in an online electronic platform.

Results: Median age at diagnosis was 56.5 (range: 15-87) years with a preponderance of females (54.6%). The most frequent primary sites were: the pancreas (31.1%), jejunum-ileum (24.2%), stomach (13.7%) and rectum (8.5%). Data regarding hormonal status was not available in most patients (82.3%). Stratified by the tumor grade (WHO 2010 classification), we observed 64.0% of NET G1, 24.7% of NET G2 and 11.3% of NEC. Poorly differentiated tumors occurred mainly in older patients (p=0.017), were larger (p<0.001), and presented more vascular (p=0.004) and lymphatic (p=0.001) invasion. At the time of diagnosis, 44.4% of GEP-NEN presented metastatic disease. Surgery (79.6%) and SA (30.7%) were the most frequently used therapies of GEP-NEN with reported grading.

Conclusion: In general, Portuguese patients with GEP-NENs presented similar characteristics to other populations described in the literature. This cross-sectional study represents the first step to establish a national database of GEP-NENs that may aid in understanding the clinical and epidemiological features of these tumors in Portugal.

3.1.2 Introduction

NEN are a heterogeneous group of rare malignancies originated from endodermal cells with secretory capacity within the neuroendocrine system. GEP-NEN represent a subtype of these tumors, located either in the pancreas or in the gastrointestinal tract (Oberg and Eriksson 2005). Although the incidence is low, it has been increasing significantly in the recent years; the age-adjusted incidence rate increased 6.4-fold from 1973 (1.09 per 100,000 persons) to 2012 (6.98 per 100,000 persons) (Dasari, Shen et al. 2017). Due to the long survival rate of patients with these tumors, the estimated 20-year limited-duration prevalence of NENs in the USA on January 1, 2014, was 171,321 (Dasari, Shen et al. 2017). The long survival reflects, besides the intrinsic biologic characteristics of neuroendocrine cells, the advances in diagnostic techniques and the awareness among clinicians (Fraenkel, Kim et al. 2014).

NEN can be classified into functional and non-functional tumors according to the presence or absence of clinical symptoms associated with hormone overproduction (Klimstra, Modlin et al. 2010). Nonspecific symptoms are evident in the majority of non-functional cases resulting in a delay in diagnosis. NEN have been a subject of long debate regarding nomenclature, grading and classification. The 2010 WHO classification, developed together with the ENETS, presented a significant progress by using two separate and complementary classification tools: histologic grading and site specific staging system, classifying NEN according to the proliferation index (fraction of Ki-67 staining or number of mitotic counts) into grade 1 (G1), grade 2 (G2) and NEC (Lombard-Bohas, Mitry et al. 2009). In 2017, this WHO classification was updated and the NEN are now divided in 3 main categories: MiNEN; NET G1/G2/G3 (WD GEP-NEN); and NEC

(poorly differentiated, large or small cell subtypes). The main differences in comparison with the 2010 classification are: the Ki-67 index of NET G1 tumors that was altered to less than 3% (instead of ≤ 2%) and an additional NET G3 sub-category that was added to the WD NEN, with a labelling index of more than 20% for Ki-67 or more than 20 mitotic counts per 10 high power field (HPF). NEC (poorly differentiated carcinomas) also require a Ki-67 proliferative index higher than 20%, as well as more than 20 mitotic counts per (santé and cancer 2017).

The aims of the available treatment options are to promote symptoms relief, improve life quality, and ideally, a disease-free setting in patients, which is largely dependent on PT size and localization. These therapies, vary from conservative procedures to pharmacologic and surgical management, and patterns of care differ between hospitals and countries depending on medical teams, experience and available resources.

Due to paucity of data on GEP-NEN in Portugal, the Neuroendocrine Tumors Study Group (GE-TNE) of SPEDM, sought to perform an observational study to present an outline of GEP-NEN patients followed at the main Portuguese hospitals regarding their socio-demographic and clinic profile (spectrum of symptoms at presentation, methods used in the diagnosis and treatment modalities applied). These data will contribute towards the effort of developing a National Registry for effective monitoring of NEN, and emphasize its importance, as well as the need for multidisciplinary involvement for a comprehensive management of GEP-NEN in Portugal.

3.1.3 Aims

The Neuroendocrine Study Group of the Portuguese Endocrinology, Diabetes and Metabolism Society sought to perform an observational study to present an outline of GEP-NEN patients followed at the main Portuguese hospitals regarding their socio-demographic and clinic profile (spectrum of symptoms at presentation, methods used in the diagnosis and treatment modalities applied).

3.1.4 Materials and Methods

We designed a cross-sectional multicenter evaluation of patients diagnosed with GEP-NEN in 15 Portuguese centers that agreed to participate in the study. Inclusion criteria were patients with more than 18 years-old of age, a confirmed diagnosis of GEP-NEN based on histopathological, cytological and/or biochemical/nuclear imaging findings; and a signed informed consent for study inclusion. Patients were consecutively enrolled in the study as they attended their medical appointment during a continuous 18-month period of the study. At the time of enrollment, data were collected directly from patients and from clinical files and submitted to an electronic platform. Variables included: age, gender, GEP-NEN subtype, site of the PT, WHO 2010 grading classification, tumor stage at diagnosis, symptoms at presentation, diagnostic procedures, hormonal and biochemical evaluations, treatment procedures, and duration of follow-up. Carcinoid syndrome was defined as values of 5-Hydroxyindoleacetic acid (5-HIAA) equal or greater than twice the upper limit of normal range and plus flushing and/or diarrhea. Insulinoma diagnosis was based on hypoglycemic symptoms, Whipple Triad and/or a positive 72-hours prolonged fasting test. Gastrinoma diagnosis was based on clinical picture and gastrin levels greater than ten times the upper limit of normal range, after excluding chronic atrophic gastritis and PPI (proton pump inhibitors) use. Imagiological procedures were evaluated according to PT location. Tumor stage was classified as localized (confined to the organ of origin), regional (invasion of the surrounding organs or tissues or regional lymph nodes) or distant (spread to distant organs). Ethical principles concerning ESP-GPP (Expanded Scope of Practice – Good Pharmacy Practicing), Helsinki Declaration and National Legislation requirements were fulfilled.

Statistical analysis was performed with SPSS® statistics (software version 15.0). Categorical and continuous variables were summarized using descriptive statistics (frequencies for categorical; mean/standard deviation or median/interquartile range for continuous, as appropriate). Proportions were

compared by the Chi-squared or Fisher's Exact test, as appropriate. Means were compared using the *t*-test or ANOVA.

3.1.5 Results

3.1.5.1. General Characteristics of the Population

A total of 314 cases were collected, whereas only 293 patients were included in the present study; the remaining 21 patients were excluded as they did not meet the inclusion criteria, such as lack of clinical information or absence of informed consent. Data are summarized in Table VII.

Table VII. Patient general characteristics

Gender (n=293)	
Male, n (%)	133 (45.4)
Female, n (%)	160 (54.6)
Age (years, n=293)	
Median (range)	59.9 (22-89)
Age at diagnosis (years, n=291)	
Median(range)	56.5 (15‡ 87)
Race (n=293)	
Caucasian, n (%)	285 (97.3)
African, n (%)	1 (0.3)
Other or not specified, n (%)	7 (2.4)
Type of diagnosis (n=293)	
Histopathological, n (%)	254 (86.7)
Cytological, n (%)	17 (5.8)
Biochemical, n (%)	5 (1.7)
Other or not specified, n (%)	17 (5.8)
Primary tumor by localization (n=293)	
Pancreas, n (%)	91 (31.1)
Head, n (%)	28 (30.7)
Body, n (%)	29 (31.9)
Tail, n (%)	32 (35.2)
Not specified, n (%)	2 (2.2)
Jejunum-ileum, n (%)	71 (24.2)
Stomach, n (%)	40 (13.7)
Type 1, n (%)	23 (57.5)
Type 2, n (%)	9 (22.5)
Type 3, n (%)	7 (17.5)
Not specified, n (%)	1 (2.5)
Rectum, n (%)	25 (8.5)
Duodenum, n (%)	20 (6.8)
Appendix, n (%)	20 (6.8)
Colon, n (%)	16 (5.5)
Oesophagus, n (%)	3 (1.0)
Unknown primary tumour	7 (2.4)
Tumor Group by Secretion	
Carcinoid Syndrome*, n positive/total studied ** (%)	17/115 (14.8)
Gastrinoma$, n positive/total studied $$ (%)	4/55 (7.3)
Insulinoma&, n positive/total studied && (%)	11/24 (45.8)
Tumor group by grade; (n=247); WHO, 2010	
NET G1, n (%)	158 (64.0)
NET G2, n (%)	61 (24.7)
NEC, n (%)	28 (11.3)
Tumor Group by Stage (n= 214); TNM (ENETs)	
Localized, n (%)	76 (35.5)
Loco Regional, n (%)	43 (20.1)
Disseminated, n (%)	95 (44.4)

*‡ Patient with 15 yo. at diagnosis, currently with 22 yo. at the time of the study; * Carcinoid Syndrome criteria: 5-HIAA > 2 times than normal value and flushing and/or diarrhea; ** Cases with 5-HIAA quantification; $ Gastrinoma criteria: gastrin ≥ 10 times than normal value and exclusion of Type I and II gastric tumours; $$ Cases with gastrin quantification; & Insulinoma criteria: hypoglycemic symptoms, Whipple Triad and/or positive 72-hours prolonged fasting test; $$ Cases with insulin quantification.*

Clinically/hormonal functional syndrome was identified in 16.5% of patients: 17 presented criteria for carcinoid syndrome, 11 for insulinoma and 4 for gastrinoma. No other hypersecreting tumors were detected in this series. The majority of cases were diagnosed by histopathology or cytopathology, 86.7% and 5.8%, respectively, and less frequently (1.7%) by biochemistry, namely, in insulinomas.

According to the WHO 2010 classification, cases where graded as NET G1 (n=158, 64.0%), NET G2 (n=61, 24.7%) and NEC (n=28, 11.3%); in 46 cases data was not available. Information regarding extension of the disease was available in 214 cases and revealed localized disease in 35.5% of cases (including gastric, duodenum and colorectal polyps) and distant disease in 44.4%. Regional spread was present in 20.1% of the cases.

The socio-demographic and clinical features of GEP-NEN patients, according to tumor grade, are summarized in Table VIII. NET G1 were more frequently detected in females (72.1%), whereas NET G2 and NEC were more common in males, 31.4% and 13.6%, respectively, (p=0.020). There was a significant association between the WHO 2010 tumor grading and age at diagnosis (p=0.017), with NEC being diagnosed at a median age of 62.5 years (range: 39 – 84) *vs.* 56.5 years (range: 32 –80) for NET G2 and 54.7 years (range: 15 – 85) for NET G1. Patients with well differentiated NEN presented a significantly higher mean body mass index (BMI) (p=0.015) in comparison with NEC patients. There was a significant association of smoking and alcohol consumption with NET G2 (p=0.007) and NEC (p=0.037). NEC patients had less co-morbidities than patients of the other two groups of NEN (57.6% *vs.* 71.4% in NET G1 and 75.8% in NET G2); these results were not statistically significant. There was a significant association between WHO 2010 tumor grading groups and PT size at diagnosis, higher in NEC (p<0.001). Vascular and lymphatic invasion were significantly more frequent in NEC (p=0.004 and p=0.001, respectively), whereas perineural invasion presented the same trend without statistical significance (p=0.064).

Table VIII. Socio-demographic and clinical features of patients, and tumor characteristics according to WHO tumor classification (grading)

	NET G1	***NET G2***	***NEC***	
Total nº of patients (n=247)	158 (64.0)	61 (24.7)	28 (11.3)	
				p
Gender (n=247)				
Male (n=118), n (%)	65 (55.1)	37 (31.4)	16 (13.6)	**0.020**
Female (n=129), n (%)	93 (72.1)	24 (18.6)	12 (9.3)	
Age (n=247), years [mean (SD)]	58.3 (12.8)	59.8 (12.7)	63.0 (12.9)	0.176
Age at diagnosis (n=246), years (median range)	54.7 (15-85)	56.5 (32-80)	62.5 (39-84)	**0.017**
Weight (n=190), Kg [mean (SD)]	71.8 (13.2)	76.9 (17.5)	68.7 (10.8)	**0.049**
BMI (n=149), kg.m^{-2} [mean (SD)]	27.0 (4.6)	28.6 (5.7)	24.6 (3.1)	**0.015**
Co-morbidities (n=231), n, *(%)*	105 out of 147, (71.4)	44 out of 58, (75.8)	15 out 26, (57.6)	0.233
Arterial hypertension (n=235), n, (%)	29 out of 150, (19.3)	5 out of 58, (8.6)	3 out of 27, (11.1)	0.139
Diabetes mellitus (n=234), n, (%)	17 out 149, (11.4)	4 out of 58, (6.9)	1 out of 27, (3.7)	0.417
Dyslipidaemia (n=239), n, (%)	15 out of 154, (9.7)	3 out of 58, (5.1)	3 out of 27, (11.1)	0.508
Cardiovascular disease (n=235), n, (%)	8 out of 150, (5.3)	2 out of 58, (3.4)	1 out of 27, (3.7)	0.897
Family history of nonendocrine neoplasm (n=167), n, (%)	51 out 105, (48.6)	22 out of 42, (52.4)	6 out of 20, (30.0)	0.254
Smoking consumption, (n=173), n, (%)	3 out of 110, (2.7)	4 out of 42, (9.5)	3 out of 21, (14.3)	**0.007**
Alcohol consumption, (n=163), n, (%)	38 out of 106, (35.8)	22 out of 37, (59.5)	10 out of 20, (50.0)	**0.037**
Tumor dimension (n=213), mm [mean (SD)]	21.3 (19.9)	32.7 (23.5)	51.7 (34.9)	**<0.001**
Vascular invasion, (n=162), n, (%)	34 out of 106 (32.1)	24 out of 41 (58.5)	9 out of 15 (60.0)	**0.004**
Lymphatic invasion, (n=155), n, (%)	39 out of 103 (37.8)	25 out of 36 (69.4)	11 out of 16 (68.7)	**0.001**
Perineural invasion, (n=119), n, (%)	26 out of 84 (31.0)	9 out of 25 (36.0)	7 out of 10 (70.0)	0.064
Hormonal status				
Functioning, (n=32) [a]	17 out of 32 (53.1)	6 out of 32 (18.6)		
Carcinoid (n=17) [b]	8 out of 17 (47.0)	5 out of 17 (29.4)		
Gastrinoma (n=4) [c]	2 out of 4 (50.0)	1 out of 4 (25.0)		
Insulinoma (n=11) [d]	7 out of 11 (63.6)	0		
Non-Functioning, (n=20) [e]	12 out of 20 (60.0)	5 out of 20 (25.0)		
MEN-1 syndrome, (n= 213) [§]	2 out of 137 (1.5)	2 out of 51 (3.9)	0 out of 25 (0.0)	0.575
Stage, (n=186)				
Localized, n, (%)	51 out of 114 (44.7)	11 out of 48 (22.9)	4 out of 24 (16.7)	**0.001**
Loco regional, n, (%)	26 out of 114 (22.8)	10 out of 48 (20.8)	2 out of 24 (8.3)	
Disseminated, n, (%)	37 out of 114 (32.5)	27 out of 48 (56.3)	18 out of 24 (75.0)	

Cases missing WHO tumour classification grading: [a] n=9; [b] n=4; [c] n=1; [d] n=4; [e] n=3.
[§] Cases reported as not presenting MEN1-syndrome clinical features (no genetic testing was performed for unsuspicious cases)

Multiple endocrine neoplasia type 1 (MEN 1) syndrome was diagnosed in 4 patients; two patients had pancreatic tumors and two patients with gastric tumors. All patients with MEN 1 syndrome had primary hyperparathyroidism and two patients had a pituitary adenoma and an adrenal adenoma, respectively.

3.1.5.2. Biochemical Tests

Biochemical data analysis concerning hormonal hypersecretion was informative in 32 patients (10.9%). Chromogranin A (CgA), equal or greater than twice the normal value was detected in 86 (51.2%) of the 168 patients evaluated (Table 3). Concerning specific markers, urinary 5-HIAA was evaluated in 115 patients and was positive in 47 (40.9%); of these, 17 patients presented carcinoid syndrome criteria. Insulinoma was identified in 11 patients (3.6%) either by Whipple's triad criteria and/or positive prolonged fasting test. Four sporadic gastrinomas were identified (Table IX).

Table IX. Biochemical tests

Biochemical tests	Positive results, n, (%)
Chromogranin A, (n=168)	86 (51.2)
5HIAA, (n=115)	47 (40.9)
Insulin, (n=25)	11 (44.0)
Gastrin, (n=55)	25 (45.5)
Glucagon, (n=8)	0
VIP, (n=9)	0
ACTH, (n=17)	0
GH, (n=12)	0

VIP, vasoactive intestinal peptide; ACTH, adrenal corticotrophin; GH, growth hormone.

3.1.5.3. Imaging Studies

The imaging modalities used as a diagnostic procedure, either for PTs or metastases, are presented in Table X.

Table X. Imaging modalities used for diagnostic procedure either for primary and metastasis

	Oesophagus	Gastric	Pancreas	Appendiceal	Duodenum	Jejunum-ileum	Colon	Rectum	UPT*	Positive/Total Exams
Upper endoscop	3/3 (100.0)	27/30 90.0)	-	-	17/19 (89.5)	-	-	-	1/4 (25.0)	48/56 (85.7)
Echoendosco py	-	7/13 (53.8)	25/28 (89.3)	-	6/6 (100.0)	-	-	10/10 (100.0)	-	48/57 (84.2)
Videocapsule	-	-	-	-	-	8/9 (88.9)	-	-	-	8/9 (88.9)
DoubleBalloon	-	-	-	-	-	1/1 (100.0)	-	-	-	1/1 (100.0)
Colonoscopy	-	-	-	-	-	12/33 (36.4)	12/12 (100. 0)	21/22 (95.5)	-	45/67 (67.2)
Entero-CT	-	-	-	-	-	4/4 (100.0)	-	-	-	4/4 (100.0)
Entero-MRI	-	-	-	-	-	11/11 (100.0)	1/1 (100. 0)	-	-	12/12 (100.0)
US scan	-	5/12 (41.7)	33/41 (80.5)	1/4 (25.0)	2/2 (100.0)	23/27 (85.2)	1/2 (50.0)	0/3 (0.0)	1/1 (100. 0)	66/92 (71.7)
CT-Scan	3/3 (100)	10/22 (45.5)	71/77 (92.2)	4/11 (36.4)	13/17 (76.5)	52/62 (83.9)	13/15 (86.7)	9/20 (45.0)	6/6 (100. 0)	181/233 (77.7)
MRI	-	0/3 (0.0)	38/44 86.4)	1/1 (100.0)	5/5 (100.0)	11/13 (84.6)	2/4 (50.0)	4/9 (44.4)	2/2 (100. 0)	63/81 (77.8)
^{111}In-pentetreotide ‡	-	6/17 (35.3)	26/36 (72.2)	2/6 (33.3)	8/12 (66.7)	25/30 (83.3)	5/8 (62.5)	2/9 (22.2)	3/3 (100. 0)	77/121 (63.6)
68Ga-PET-SSTR	-	5/12 (41.7)	26/31 (83.9)	2/5 (40%)	1/2 (50.0)	32/34 (94.1)	2/2 (100. 0)	5/11 (45.5)	2/2 (100. 0)	75/99 (75.8)
PET-FDG	2/2 (100.0)	0/5 (0.0)	10/17 (58.8)	0/1 (0.0)	1/1 (100.0)	2/4 (50.0)	3/5 (60.0)	-	1/1 (100)	19/36 (52.8)

‡ Octreoscan®
**UPT: unknown primary tumour;*
CT: computed tomography;
MRI - magnetic resonance imaging;
PET-FDG: Positron-emission tomography – (18F) fluorodeoxyglucose

CT scan was performed in 233 (79.5%) of the 293 patients and identified primary and/or metastatic tumor location in 79.5% of the evaluated cases. 111In-pentetreotide scintigraphy (Octreoscan®), was performed in 121 (41.3%) of the 293 patients and was informative in 63.6% of the evaluated cases. 68Ga-PET-SSTR scan, was used in 99 (33.8%) of the 293 patients and was informative in 75.8% of the evaluated cases. 111In-pentetreotide scintigraphy and 68Ga-PET-SSTR scan were mainly used in NET G1 and NET G2 patients, 89.8% and 93.1%, respectively. 18F-FDG-PET was evaluated in 36 (12.3%) of 293 patients. Upper gastrointestinal endoscopy presented the highest efficiency in localizing esophageal (3 out of 3, 100%), gastric (27 out of 30, 90%) and duodenal tumors (17 out of 19, 89.5%). Echoendoscopy was valuable in the detection of duodenal (6 out of 6, 100%), pancreatic (25 out of 28, 89.3%) and gastric (7 out of 13, 53.8%) tumors. Colonoscopy was the main diagnostic procedure in colonic NEN detection (12 out of 12, 100%), as well as, in rectal NEN (21/22, 95.5%). For midgut tumors, magnetic resonance imaging (MRI), CT and video capsule, were

the mostly used imaging procedures; 68Ga-PET-SSTR, demonstrated to be the most sensitive (94.1%) imaging tool.

Extension of the disease

Extension of the disease was evaluated in 186 patients (Figure 8 and Table VIII).

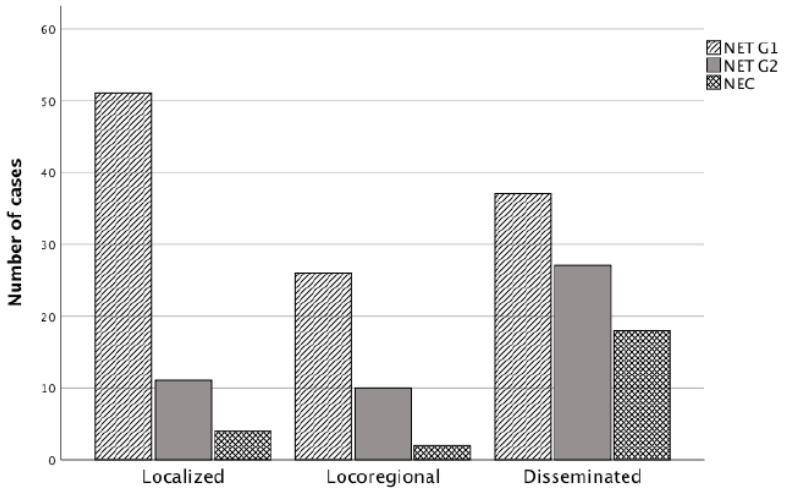

Figure 8. Extension of disease according to WHO 2010 classification

Localized disease was more frequent in NET G1 (44.7%). Regional disease was detected in 20.1% of the patients: 22.8% with NET G1, 20.8% with NET G2 and 8.3% with NEC. Metastases were present in 32.5% of patients with NET G1, in 56.3% with NET G2 and in 75.0% with NEC. Among cases with distant metastases at presentation (n=82), 30.5% presented liver metastases. Bone metastases were detected in one patient with a NET G2 and two patients with NEC. Only one patient with NEC had lung metastases. Other sites of distant metastases included the peritoneum (five patients: one NET G1, one NET G2 and three NEC), adrenal glands (one patient with NEC), ovary (one patient with NET G1) and inferior vena cava (one patient with NET G1).

3.1.5.4. Treatment procedures

Endoscopic removal of the tumors was possible in 40 patients with localized gastric, duodenal and colorectal NENs. According to the WHO 2010 classification, either curative or cytoreductive surgery was performed in 125 out of 155 cases (80.6%) of NET G1, 48 out of 60 cases (80.0%) of NET G2, and 18 out of 25 cases (72.0%) of NEC (Table XI); overall, 191 of 240 patients (79.6%) were treated with surgery. Concerning patients with disseminated disease, 22 patients (18.2%) with NET G1, 9 patients (20.5%) with NET G2 and 8 patients (44.4%) with NEC were submitted to metastatic tumor surgery, mainly liver metastasectomy.

Table XI. Treatments administered to patients with GEP-NEN

Endoscopic Therapy (n=40)	**Gastric, n (%)**	**Duodenum, n (%)**	**Rectum, n (%)**	
	21 (52.5)	4 (10.0)	15 (37.5)	
	NET G1	***NET G2***	***NEC***	***p***
Surgical Therapy (n=240) [$]	125 out of 155 (80.6)	48 out of 60 (80.0)	18 out 25 (72.0)	0.607
Surgery of Metastases (n=183) [$]	22 out of 121 (18.2)	9 out of 44 (20.5)	8 out of 18 (44.4)	0.055
TAE (n=199) [$]	7 out of 131 (5.3)	3 out of 49 (6.1)	0 out of 19 (0.0)	0.781
RFA (n=101) [$]	3 out of 61 (4.9)	1 out of 27 (3.7)	0 out of 13 (0.0)	>0.999
Systemic Therapies				
Somatostatin Analogues (n=231) [$]	31 out of 152 (20.4)	32 out of 54 (59.3)	8 out of 25 (32.0)	**<0.001**
Interferon (n=231) [$] **+ SSAs**	3 out of 152 (2.0)	1 out f 55 (1.8)	0 out of 24 (0.0)	>0.999
Target Therapies* (n=231) [$]	2 out of 153 (1.3)	2 out of 53 (3.8)	3 out of 25 (12.0)	**0.020**
PRRNT (n=230)** [$]	3 out of 150 (2.0)	6 out of 55 (10.9)	0 out of 25 (0.0)	**0.021**
Chemotherapy (n=244) [$]	3 out 157 (1.9)	6 out of 60 (10.0)	11 out of 27 (40.7)	**<0.001**

[$] Number of cases with information
TAE=Transhepatic arterial embolization;
RFA = radiofrequency ablation;
PRRNT – Peptide Receptor Radionuclide Therapy
**Sunitinib;*
***^{177}Lu-THERA.*

Although 95 patients presented liver metastases at diagnosis, loco-regional ablative therapy, such as trans-arterial embolization (TAE), trans-arterial chemoembolization (TACE), radioembolization or radiofrequency (RF) / thermoablation (TA) was only performed in 14 patients with WD GEP-NEN; 70.0% of the cases submitted to TAE and 75.0% submitted to RF/TA were NET G1. Only four patients were submitted to radioembolization, being three NET G1 and one NET G2.

Systemic therapy included SA, interferon-α2b, target therapies with tyrosine kinase inhibitors and mTOR inhibitors, peptide receptor radionuclide therapy (PRRT) and chemotherapy (Table IX, Fig. 9).

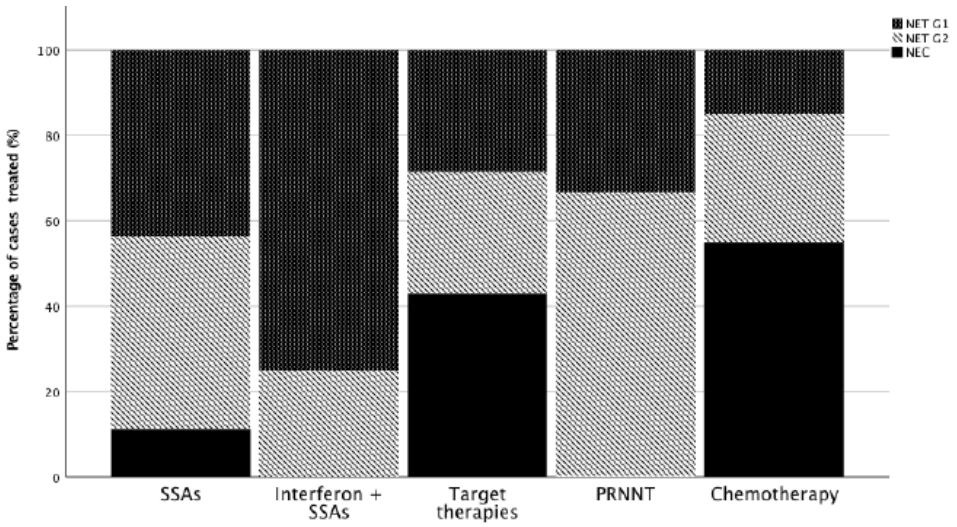

Figure 9. Cases submitted to different systemic therapies according to WHO 2010 classification

SA were mostly used in WD GEP-NEN (p<0.001), comprising 20.4% of NET G1, 59.3% of NET G2, and 32.0% of NEC. Only 4 patients received combined treatment with SAs and interferon-α2b. Target therapies as sunitinib and everolimus were used in seven (3.0%) patients, two with NET G1, two with NET G2 and three with NEC. PRRT was used in nine (3.9%) of the patients, mainly WD GEP-NEN (33.3% NET G1 and 66.7% NET G2). Chemotherapy treatment was performed in 20 patients, mostly in NEC of the colon and the pancreas (11 patients; p<0.001).

3.1.6 Discussion

GEP-NEN have been historically considered a rare and heterogeneous group of neoplasms. They comprise approximately 0.5% of all human cancers, and 2% of gastrointestinal tumors (Oberg and Eriksson 2005). New data from SEER 18 (Dasari, Shen et al. 2017) reported a 6.5-fold increase in the annual incidence from 1973 to 2012 in NEN (Dasari, Shen et al. 2017), reinforcing the need for research in this field. GEP-NEN often exhibit relatively indolent clinical courses, a delay in the diagnosis and tend to present metastases at the time of diagnosis, preserving the potential for lethal progression.

The present study was designed to characterize the overall scenario of GEP-NENs in Portugal, namely, the incidence and epidemiology of these tumors, socio-demographics and clinical profiles of the patients and the patterns of care in a multicenter audit. Our results provide a comprehensive and relevant information on a group of neoplasm still poorly characterized, particularly, in Southern Europe. Published data from GEP-NEN in European countries is available in a French registration study (Lombard-Bohas, Mitry et al. 2009), in a Spanish study of the Neuroendocrine Tumors Study Group Registry of Spain (RGETNE) (Garcia-Carbonero, Capdevila et al. 2010), in an Italian epidemiological study (Faggiano, Ferolla et al. 2012), in a prospective Greek registry (Nikou, Pazaitou-Panayiotou et al. 2016), and in the United Kingdom and Northern European countries (Hemminki and Li 2001, Lepage, Rachet et al. 2007, Hauso, Gustafsson et al. 2008, Yao, Hassan et al. 2008). Worldwide, the most characterized cohorts are from the United States of America (USA) (Yao, Hassan et al. 2008, Dasari, Shen et al. 2017), and there is data available from Asian countries, such as China (Zhang, Ma et al. 2014) and Japan (Ito, Sasano et al. 2010). Overall, our findings are in accordance with reports of NEN from other countries and corroborate that they are a heterogeneous group of tumors with a wide range of clinical presentation. We observed a similar gender ratio with a slight preponderance for females, as observed in USA series (Hauso, Gustafsson et al. 2008), Canadian series (Hallet, Law et al. 2015) and in an Italian study (Faggiano, Ferolla et al. 2012). In our series, the pancreas was the most frequent PT site, followed by the jejunum-ileum and the stomach. These findings

are in agreement with data from Southern European countries, as the Italian and Greek cohorts (Faggiano, Ferolla et al. 2012, Nikou, Pazaitou-Panayiotou et al. 2016) as well as in China (Fan, Zhang et al. 2017), but in contrast with other published studies (Lombard-Bohas, Mitry et al. 2009, Garcia-Carbonero, Capdevila et al. 2010, Hallet, Law et al. 2015, Dasari, Shen et al. 2017), where the gastrointestinal tract was reported as the most frequent primary site. These inconsistencies may be due to a referral bias and may suggest geographic and ethnic variation in the carcinogenesis of GEP-NEN. A recent publication stresses the differences in geographic and ethnic distribution, other than NEN fortuitous location and identification related to the current accuracy of the diagnostic methods (Huguet, Grossman et al. 2017), and points to the possibility of involved environmental RFs. Prospective and larger studies will be useful to further clarify these findings.

The present study provides a comprehensive report on diagnostic and therapeutic procedures used in current clinical practice in Portugal. Like the Spanish results reported by the RGETNE, in Portugal there is a limited overall use of biochemical tests at diagnosis, namely the general marker serum chromogranin A or urinary 5-HIAA quantification for midgut tumors.

In our cohort, as in another series (Garcia-Carbonero, Capdevila et al. 2010), the most frequent functioning tumor were NEN with carcinoid syndrome, followed by insulinoma, and apparently sporadic gastrinoma. No glucagonoma, VIPoma, somatostatinoma or other rare syndromes were identified. It should also be taken into consideration that in 71.7% of the cases, the hormonal secretion by the tumor was not evaluated. This seems to reflect a low referral rate of patients to specialized centers, low participation of endocrinologists in the oncology team and/or a limited laboratory support in some of the institutions that participated in this study. Our results highlight the ongoing demand for an adequate management of diagnostic, treatment and follow-up work-out for patients with GEP-NEN. Most of the international epidemiological studies report data about localization, histological classification and staging of GEP-NEN but information about their hormonal secretion is scarse. Biochemical evaluation is important, not only for diagnostic purposes but also for therapeutic decision and monitoring of treatment responses, and an adequate assessment of tumor secretion is strongly

encouraged. Genetic testing is also important when clinically indicated, as it allows for: 1) a personalized life-long screening for prototypic tumors and their timely treatment; 2) the identification of affected family members that may benefit from this screening; and 3) appropriate genetic counseling. In our series, the majority of the cases lacked genetic evaluation for clinical suspicion of hereditary syndromes.

Histological classification of NEN is evolving as the WHO revised the nomenclature and classification of GEP-NENs in 2010 [5] and updated it in 2017 [6]. Histopathological characterization with immunohistochemistry markers such as chromogranin and synaptophysin are essential to make the diagnosis. Mitotic index and/or immunohistochemistry for Ki-67 labelling index are mandatory to generate the tumor grading (Klimstra, Modlin et al. 2010); these are minimum requirements for an accurate pathological classification. At the time of the inclusion of the patients in the present study, the histological classification was performed according to the 2010 WHO criteria, the up-to-date guidelines used for this study. Overall, in this study the frequency of NET G1, NET G2 and NEC fit with other reports.

Tumor metastases at diagnosis represents an important prognostic marker (Dasari, Shen et al. 2017). In this series, distant metastases were detected in 44.4% of patients (NET G1: 32.5%; NET G2: 56.3%; and NEC: 75.0). This is consistent with other studies, as the Spanish and Italian studies (Garcia-Carbonero, Capdevila et al. 2010, Faggiano, Ferolla et al. 2012), where distant metastases were observed in 44% and 42% of patients, respectively, and contrasts with a lower rate of distant metastases at diagnosis in the Greek (Nikou, Pazaitou-Panayiotou et al. 2016), Chinese (Zhang, Ma et al. 2014), and the Canadian (Hallet, Law et al. 2015) studies (25.0%, 6.0% and 20.8%, respectively), as well as the SEER Registry (21.0%) (Yao, Hassan et al. 2008). An explanation for these differences may be due to the inclusion of cases from oncological institutions, where the proportion of metastatic disease is considerably higher. In this study, the oncological institutions, from Lisbon and Porto contributed with 46% of the patients included.

Endoscopic therapy is the mainstay for type 1 and 2 gastric endocrine neoplasia and for localized duodenal and colorectal NEN. In this cohort, endoscopic therapy was performed mainly in those cases. Surgery remains the treatment of choice for GEP-NEN, with curative intent whenever feasible. If tumor is unresectable, several approaches are available to induce tumor debulking as a manner to control life-threatening symptoms due to hormone secretion and to increase patient survival and quality of life (Keutgen, Nilubol et al. 2016, Guo, Zhang et al. 2017). In experienced centers, ablative therapies are a good option to treat liver metastatic disease (Guadagni, Fiorentini et al. 2017). Our results show that either primary or cytoreductive surgery was performed in the majority of the hospitals included and mainly in well differentiated NEN. Ablative therapies were used in less than 5% of the patients probably due to the fact that few centers have this treatment available. This finding indicates the need of referral of the patients to centers where they can benefit from these therapeutic options.

Currently, the standard of care for systemic treatment in advanced NEN treatment are SA, proved to be effective in controlling excessive hormonal secretion (Moertel 1987, Oberg, Kvols et al. 2004) and allowing long-term improvement in secretory symptoms in 30–70% of patients. Recent studies report an additional anti-proliferative role of SA in non-functioning midgut (Oberg, Kvols et al. 2004, Rinke, Muller et al. 2009), pancreatic and lung NENs (Caplin, Pavel et al. 2014), reflected in the significant progression free-survival in the treated patients when compared with placebo. Other therapeutic options include biologic agents interfering with specific molecules of cell signaling pathways, *e.g.* mTOR and VEGF, with everolimus and sunitinib, respectively, both approved for pancreatic NEN (Raymond, Dahan et al. 2011, Yao, Shah et al. 2011). Everolimus was also approved for the treatment of advanced non-functioning lung and gastrointestinal NEN (Yao, Fazio et al. 2016). Studies using oral chemotherapy with temozolomide and capecitabine are demonstrating promising results in well differentiated pancreatic NEN (Strosberg, Fine et al. 2011). However, classic cytotoxic drugs still continue to be the first-line therapy for poorly differentiated GEP-NEN and are effective (up to 60% response rates) in well differentiated pancreatic NEN; however, early relapses often occur (Garcia-Carbonero, Rinke et al. 2017). Concerning the therapeutic options in the present study, endoscopic

therapies, either curative or cytoreductive surgery and SA treatment were the preferred options for the majority of patients. SA were the most frequently used drugs in our study. Locoregional ablative, PRRT and target therapies were rarely used. Remarkably, PRRT was more frequently chosen than target therapies. This fact was remarkable, as in the Portuguese National Health System only one center offered this therapeutic modality at the time of the present study. As in other series and according to the guidelines, chemotherapy was the treatment of choice in NEC, and was also an option in well differentiated non pancreatic NEN, which may reflect the inclusion of older cases and/or the absence of referral to specialized centers.

The results obtained in this study represent the first comprehensive registry of GEP-NEN in Portugal performed by the Neuroendocrine Study Group of the Portuguese Society of Endocrinology, Diabetes and Metabolism. It provides a valuable insight into the epidemiology, current clinical practice and therapy strategies of this heterogeneous disease and will set the ground for the development of a National Registry of NEN. It reinforces the need for a national clinical framework for GEP-NEN, in order to ensure a systematic surveillance of the disease and ultimately improve the diagnosis, clinical management and outcome of NEN patients.

3.2 Visceral Obesity and Metabolic Syndrome are Associated with Well-Differentiated Gastroenteropancreatic Neuroendocrine Neoplasia

3.2.1 Abstract

The determinants for GEP-NEN`s recent burden are matter of debate. Obesity and MetS are well established risks for several cancers even though no link with GEP-NEN was yet established. Our aim in this study was to investigate whether WD GEP-NEN were associated with obesity and MetS. Patients with WD GEP-NEN (n = 96) were cross-matched for age, gender, and district of residence with a control group (n = 96) derived from the general population in a case-control study. Patients presented gastro-intestinal (75.0%) or pancreatic (22.9%) tumors, grade G1 (66.7%) or G2 (27.1%) with localized disease (31.3%), regional metastasis (16.7%) or distant metastasis (43.8%) at diagnosis, and 45.8% had clinical hormonal syndromes. MetS was defined according to JIS criteria.

WD GEP-NEN were associated with MetS criteria as well as the individual components' WC, fasting TG, and FPG ($p = 0.003$, $p = 0.002$, $p = 0.011$ and $p < 0.001$, respectively). The likelihood of the association was higher when the number of individual MetS components was greater than four. MetS and some individual MetS components including visceral obesity, dyslipidemia, and increased FPG are associated with WD GEP-NEN.

This data provides a novel insight in unraveling the mechanisms leading to GEP-NEN disease.

3.2.2 Introduction

GEP-NEN are considered a rare entity even though a 6.5-fold increase in incidence was observed in the past four decades (Dasari, Shen et al. 2017), which are believed to be predominantly driven by the rising number of the incidental detection of low-stage tumors (McMullen, Al-Jahdali et al. 2017). GEP-NEN are currently the second most frequent digestive tumor only surpassed by colorectal cancer (Yao, Hassan et al. 2008). Grounded on the increasing knowledge related to the biology of the tumors accumulated in the past two decades, a great effort has been made in order to establish guidelines for GEP-NEN classification and management (O'Toole, Kianmanesh et al. 2016, Pavel and de Herder 2017). Nevertheless, despite the fact that significant advances were made towards the understanding of the genetics and molecular mechanisms associated with NEN, very little is known about the etiology of sporadic tumors or the reasons for the rising incidence observed over the past several decades (Pavel and de Herder 2017). The possible link between obesity and cancer was first described in the 1940s even though the molecular mechanisms underlying this association were only recently described (Vigneri, Frasca et al. 2009, Byers and Sedjo 2015). Obesity is frequently associated with IR, which is related to a state of systemic and local low grade chronic inflammation responsible for the activation of a number of signaling pathways involving hormone control, cell proliferation, and immunity (Vigneri, Frasca et al. 2009, Byers and Sedjo 2015) that lead to neoplastic transformation of cells. IR, MetS and T2-DM are now well-established RFs for many cancers including postmenopausal breast cancer, endometrial cancer, colorectal cancer, and hepatocarcinoma (Arcidiacono, Iiritano et al. 2012). Chronic inflammation is also a well-recognized cancer promoter (Rakoff-Nahoum 2006) such as chronic pancreatitis that leads to pancreatic cancer (Gukovsky, Li et al. 2013), ulcerative colitis to colon cancer (Scarpa, Castagliuolo et al. 2014), and non-alcoholic steatohepatitis (NASH) for liver cancer (Dongiovanni, Romeo et al. 2014).

Whether obesity and MetS could be involved in the etiology of GEP-NEN to the extent of justifying the recent burden of the disease is unknown. This applies in

particular to WD GEP-NEN, corresponding to the WHO 2010 grade G1 and G2, which have a natural history dramatically different from NEC (Heetfeld, Chougnet et al. 2015).

3.2.3 Hypothesis and Aims

The aim of the current study was to evaluate the possible association between MetS and MetS individual components with WD GEP-NEN by performing a case-control study comparing data from patients from a large tertiary cancer center with a matched control group derived from the background general population.

3.2.4 Materials and Methods

Patients with confirmed WD GEP-NEN (n = 96) were recruited from the endocrine tumors clinic of a large tertiary referral center for oncological diseases. The inclusion criteria were a confirmed diagnosis of WD GEP-NEN by histopathology and/or ^{68}Ga-PET-SSTR. The exclusion criteria were under 18 years of age when first diagnosed, familial GEP-NEN, NEC, and T1-GEN since these tumors have well-established etiology and distinctive behavior (Heetfeld, Chougnet et al. 2015, Benafif and Eeles 2016, Delle Fave, O'Toole et al. 2016).

From a total number of patients recruited with confirmed WD GEP-NEN (n = 120) that consented to participate in the study, those who did not fulfil the inclusion criteria or had insufficient data for analysis were excluded (n = 24). The remainder of patients (n = 96) were then matched for age, gender, and district of residence with a control group (n = 96) of the general population derived from the PORMETS study, which is a nationwide epidemiological study designed to assess the prevalence of MetS in the general population (Raposo, Martins et al. 2017, Raposo, Severo et al. 2017, Raposo, Severo et al. 2018). The present study was approved by the National Data Protection Committee (CNPD 4906/2015) as well as the Institutional Ethics Committee (IPO 366/2013).

Patients gave their written informed consent to participate and were consecutively enrolled as attending routine clinic appointments.

Data for analysis was collected through a face-to-face patient interview to assess the past medical history of T2-DM, hypertension, dyslipidemia, ongoing medications, and family history of T2-DM while height, weight, WC, and BP measurements were collected directly or indirectly, according to medical practice standards. Most patients were newly diagnosed WD GEP-NEN patients who were referred to our center and the parameters used for the assessment of MetS refer to the time of diagnosis. For patients with longer disease duration referred to our center after treatment initiation (surgery or somatostatin analogues), data was retrieved from patient digital records from other institutions (hospital or general practice registries) to ensure a minimum bias.

Biochemical data including FPG and the lipid profile were evaluated while off any active anti-tumor treatment. The only exception was for FPG and fasting plasma insulin measurements that were used for Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) calculation, which were assessed while on SA in those patients who were already under oncological treatment. WD GEP-NEN were classified according to PT localization, the presence of the hormone secretion syndrome, the WHO 2010 grading system, and disease extension (ENETS TNM staging system) (Rindi, Kloppel et al. 2006, Rindi, Kloppel et al. 2007). Cases with insufficient data to allow grading were classified as WD GI-NEN if found to express somatostatin receptors on ^{68}Ga-PET-SSTR (n = 6). Patients with metastatic tumors and carcinoid syndrome without any visible pancreatic or thoracic lesions on imaging investigations were classified as having a midgut WD GEP-NEN (*n* = 2). No insulinoma or rare functional panNEN presenting with hyperglycemia such as glucagonoma, VIPoma or somatostatinoma were included in this study series.

Patients were classified into three categories according to the BMI, which included normal weight (BMI < 25 Kg/m^2), overweight (BMI 25–29.9 Kg/m^2), or obese (BMI ≥ 30 Kg/m^2) (Borrell and Samuel 2014) and according to FPG levels into normoglycemic (NG, FPG < 100 mg/dL) and impaired fasting glucose (IFG 100–126 mg/dL) or T2-DM (T2-DM, FPG ≥ 126 mg/dL) (American Diabetes

2010). MetS was classified, according to the JIS of NHLBI/AHA/WHF/IAS/IASO criteria (Alberti, Eckel et al. 2009): WC ≥ 88 cm (female) or 102 cm (male), BP ≥ 130 mmHg and/or 85 mmHg or previous history of high BP or under BP lowering medication. HDL-c < 40 mg/dL (male) or < 50 mg/dL (female) or drug treatment for reduced HDL-c, TG ≥ 150 mg/dL or under TG lowering drugs, and FPG ≥ 100 mg/dL or ongoing glucose-lowering drug treatments.

Insulin was determined by an automated enzyme-labeled chemiluminescent immune metric solid-phase assay (IMMULITE 2000). IR was assessed by HOMA-IR index calculated using the formula fasting plasma insulin (FPI) (μU/mL)/FPG (mg/dL)/405. IR cut-offs were based on Matthews definition (Matthews, Hosker et al. 1985): <3 (insulin sensitive), ≥ 3 < 5 (IR) and ≥ 5 (severe IR).

Statistical analysis was performed using PASW 18.0. Categorical and continuous variables were summarized using descriptive statistics (frequencies for categorical, mean/standard deviation or median/interquartile range for continuous, as appropriate). Proportions were compared by the Chi-squared or Fisher Exact test. Means were compared using the t-test or ANOVA while medians were compared using the Mann-Whitney or Kruskal-Wallis tests. Unconditional logistic regression models were used to evaluate the odds of developing GEP-NEN, according to weight, glucose abnormalities, IR, and MetS criteria. A level of significance of 0.05 was adopted.

3.2.5 Results

3.2.5.1. Patients' Characteristics

Table XII provides the demographic, anthropometric, and clinical features of WD GEP-NEN patients and controls. Patients' mean age at WD GEP-NEN`s diagnosis was 58.2 years and 62.4 years at the time of a study assessment. There was a slight preponderance of males (52.1%) and the majority of the patients lived within the area of our institution (45.8%).

Table XII. Demographic, anthropometric, clinical, and biochemical features of patients with WD GEP-NEN and controls

	Patients (n=96)	Controls (n=96)	p
Age in years - mean (SD)	62.4 (11.20)	62.4(12.1)	0.979
Age at Diagnosis in years - mean (SD)	58.2 (11.2)	-	-
Duration of the disease in months - mean (SD) ; (n=92)	55.3 (37.5)	-	-
Gender – n (%)			0.772
Male	50 (52.1)	52 (54.2)	
Female	46 (47.9)	44 (45.8)	
METABOLIC TREATMENT			
Previous anti-hypertensive treatment (n=95/71)	48 (50.5)	12 (16.9)	<0.001
Previous anti-dyslipidemia treatment (n=95/71)	36 (37.9)	7 (9.9)	<0.001
Statins	33 (91.7)	6 (8.5)	
Fibrates	3 (8.3)	3 (3.2)	
Previous anti-diabetic treatment (n=79)	12 (14.2)	3 (4.2)	0.102
Insulin sensitizers	7(58.3)	3 (4,2)	
Sulfonylureas	2(16.7)	-	
Insulin	3 (25.0)	-	
CLINICAL EVALUATION			
Height, cm - median(IQR))	164,0 (14,5)	163,0 (39,0)	0.573
Weight, cm - mean (SD)	72,6 (13,6)	72.0 (13,3)	0.753
BMI, Kg/m^2 - mean (SD)	26,9 (4,2)	27,2 (4,1)	0.645
WC, cm - mean (SD)	94.9 (12.0)	93.0 (10.6)	0.236
SBP, mmHg - median (IQR)	135.0 (21.0)	130.0 (28.0)	0.247
DBP, mmHg - median (IQR)	75.5 (17.0)	70.5 (12.0)	0.203
BIOCHEMICAL EVALUATION			
TC, mg/dL - mean (SD)	192.1 (44.4)	208.1 (49.8)	0.020
LDL-c, mg/dL - mean (SD)	114.1 (37.1)	139.6 (41.0)	<0.001
HDL – c, mg/dL - mean (SD)	50.8 (13.1)	44.8 (12.3)	0.001
TG , mg/dL - median (IQR)	117.5(78.5)	105.0 (77)	0.091
FPG , mg/dL - median (IQR)	101.0 (22.0)	88.5 (27.5)	<0.001
FPI ((median(IQR))	6.2 (5.0)	5.8 (6.0)	0.372
HOMA-R (median(IQR))	1.4 (1.6)	1.4 (1.6)	0.274

BMI (body mass index); WC (waist circumference); SBP (systolic blood pressure); DBP (diastolic blood pressure); TC (total cholesterol); TG (triglycerides); FPG (fasting plasma glucose); FPI (fasting plasma insulin); HOMA-IR (homeostasis model assessment insulin resistance).

Most patients had previous diagnosis of hypertension (63.5%), dyslipidemia (62.3%), or T2-DM (17.7%). Family history of T2-DM was present in 48.1% of cases. A large percentage of patients were under BP lowering drugs (50.5%), lipid lowering medications (37.9%) including statins (91.7%), and glucose lowering therapy (14.2%) including dipeptidyl peptidase-4 (DPP-4) inhibitors and/or metformin (58.3%), sulfonylureas (16.7%), or insulin (25.0%). Although

there was no significant difference between WD GEP-NEN patients and controls concerning the use of glucose lowering therapy, the proportion of patients under BP or lipid lowering therapy was significantly higher in patients than in controls ($p < 0.001$). There were no significant differences between patients and controls concerning weight, BMI, systolic BP (SBP), diastolic BP (DBP), FPI, and HOMA-IR. Total cholesterol (TC) and LDL-c levels were significantly higher ($p = 0.02$ and $p < 0.001$, respectively) and HDL-c was significantly lower ($p = 0.001$) in controls when compared to patients. Fasting plasma glucose (FPG) was significantly higher in patients than in controls ($p < 0.001$) despite the fact that 14.2% of the patients were under glucose lowering therapy.

Subgroup analysis of patients comparing those that were under SA treatment with those that were not (Table XIII) did not show any significant differences between the two groups regarding MetS ($p = 0.746$), WC ($p = 0.198$), TG levels ($p = 0.503$), HDL-c ($p = 0.786$), FPG ($p = 0.862$), FPI ($p = 0.187$), and HOMA-IR ($p = 0.438$).

Table XIII. Comparison of anthropometric and biochemical metabolic profile of WD GEP-NEN patients under somatostatin analogues (SA positive) treatment versus patients with no somatostatin analogue exposure (SA negative)

	SA+ (n=60)	SA- (n=36)	p
WC (mean/SD)	96.2 (12.4)	96.7 (11.3)	0.198
TG ((median(IQR))	121.5 (73.3)	111.0 (91.5)	0.503
HDL ((median(IQR))	50.6 (13.3)	55.7 (12.9)	0.786
FPG ((median(IQR))	102.0 (22.0)	99.5 (20.0)	0.862
FPI ((median(IQR))	6.1 (4.0)	7.3 (9.0)	0.187
HOMA-R (median(IQR))	1.4 (0.8)	1.5 (2.1)	0.438

WC (waist circumference); TG (triglycerides); Fasting Glucose (FPG); FPI (fasting plasma insulin); HOMA-IR (homeostasis model assessment insulin resistance)

The most frequent localization of the PT was gastrointestinal in 75% of cases (60.0% in the ileum, 40% non-ileum), which was followed by panNEN that represented 22.9% of cases while, in two cases, the PT localization was unknown.

The tumor's hormone secretion profile was determined in the majority of the patients (90.6%) while 45.8% were found to be secreting tumors presenting with carcinoid syndrome (93.2%) or sporadic gastrinomas (6.8%). WD GEP-NEN were either grade G1 (66.7%) or G2 (27.1%) tumors. At presentation, 43.8% of patients were found to have distant metastasis, 16.7% of patients had loco-regional disease, and 31.2% of patients had localized disease, which included duodenal and colorectal NEN polyps. Patients without distant metastasis referred to our center after surgical removal of the PT without information concerning available lymph nodes were considered to have an undetermined tumor stage (n = 8). WD GEP-NEN patients were treated in accordance with established treatment guidelines with SA (62.5%), liver ablative therapies including hepatic arterial embolization (TAE), radiofrequency (RF) and thermal ablation (TA) (29.5%), or with PRRT with 177Luthetium-DOTATATE in 7.0%. Only one of the patients included was submitted to chemotherapy and no patients went on target therapies (Table XIV).

Table XIV. WD GEP-NEN patients characteristics

Localization of PT (n=96)	**n(%)**
GI-NEN	72 (75.0)
Jejunum-ileum	45 (62.5)
duodenum	10 (13.9)
rectum	8 (11.1)
appendix	5 (6.9)
colon	2 (2.8)
stomach	1 (1.4)
ampulla	1 (1.4)
panNEN	22 (22.9)
Unknown (UK)	2 (2.1)
Hormonal syndrome (n=96)	
Yes (93.2% carcinoid syndrome; 6.8% gastrinoma)	44 (45.8)
No	43 (44.8)
Unknown (UK)	9 (9.4)
Grading (WHO 2010) - n=96	
NETG1	64 (66.7)
NETG2	26 (27.1)
Unknown (UK)	6 (6.3)
Staging (ENETS) - (n=96)	
Local disease	30(31.3)
Loco regional disease	16 (16.7)
Disseminated disease	42 (43.8)
Unknown (UK)	8(8.3)
PAST HISTORY	
Family History of T2-DM (n=81)	39 (48.1)
Hypertension (n=96)	61 (63.5)
Dyslipidemia (n=96)	60 (62.5)
T2-DM (n=96)	17 (17.7)
NEN Treatment	
Endoscopic Therapy (n=95)	11(11.6)
Surgery (n=96)	73 (76.8)
SA (n= 95)	60 (62.5)
Liver ablative therapies (n= 95)	28(29.5)
PRRT (n=95)	7 (7.4)
Chemotherapy (n=96)	1 (1.0)
Target therapies (n=96)	0 (0.0)

PT (primary tumor); GI-NEN (gastrointestinal neuroendocrine neoplasia); panNEN (pancreatic neuroendocrine neoplasia); T2-DM (type 2 diabetes mellitus); SA (somatostatin analogues); PRRT (Peptide Receptor Radionuclide Therapy).

3.2.5.2. WD GEP-NEN association with Obesity, Glucose Abnormalities, MetS and IR

A strong association between WD GEP-NEN and MetS (p=0.003) and MetS individual JIS criteria such as WC (p=0.002), fasting TG (p=0.011), FPG (p<0.001), as well as a moderate association with severe IR (p=0.014) was found (Table XV).

Table XV. Association of MetS, MetS components, and IR with WD GEP-NEN and controls

	Pts. n(%)	Controls n(%)	OR (95%CI)	p
Obesity Classification				
Normal weight (BMI<25 Kg/m2)	31 (32.3)	33 (34.4)	1	
Excess weight (BMI≥ 25< 30 Kg/m2)	41(42.7)	41 (42.7)	1.1 (0.6-2.0)	0.851
Obesity (BMI≥ 30Kg/m2)	24(25.0)	22(22.9)	1.2 (0.5-2.5)	0.847
Classification of Glucose Abnormalities				
Normal	62 (64.6)	71 (75.5)	1	
IFG	14 (14.6)	4 (4.3)	4.0(1.3-12.8)	0.013
T2-DM	20(20.8)	19 (20.2)	1.2 (0.6-2.5)	0.608
Metabolic Syndrome and Components				
WC ≥ 88 (F) /102 (M) cm	55 (58.9)	34 (35.8)	2.5 (1.4-4.6)	0.002
BP ≥ 130/85 mmHg (or anti-hypertensive drugs)	63(65.6)	61 (64.2)	1.06 (0.6-1.9)	0.838
C-HDL< 50 (F)/40 (M) mg/dL (or anti-*dyslipidemia* drugs)	52(54.2)	48 (50.5)	1.6 (0.7-2.0)	0.615
TG ≥ 150 mg/dL (or anti-dyslipidemia drugs)	41(42.7)	24 (25.3)	2.2 (1.2-4.1)	0.011
FPG ≥ 100 mg/dL (or hypoglycemic drugs)	53 (55.2)	21 (22.1)	4.3 (2.3-8.2)	<0.001
Metabolic Syndrome	58 (60.4)	37 (54.4)	2.4 (1.3-4.3)	0.003
IR Classification n (%)*				
Insulin sensitive (HOMA-IR <3)	54 (56.3)	80 (85.1)	1	
Insulin Resistant (HOMA-IR ≥3<5)	2 (3.0)	10 (10.6)	0.3 (0.1-1.4)	0.131
Very insulin resistant (HOMA-IR ≥5)	11 (11.5)	4 (4.3)	4.1 (1.2-13.5)	0.014

WC (waist circumference); BP (blood pressure); TG (triglycerides); FPG (fasting plasma glucose); MetS (metabolic syndrome); BMI (body mass index); IFG (impaired fasting glucose); T2-DM (type 2 diabetes mellitus); IR (insulin resistance); HOMA-IR (homeostasis model assessment insulin resistance).

Moreover, the association increased significantly if four or five MetS individual components were present (p = 0.024 and p = 0.032, respectively) (Figure 10).

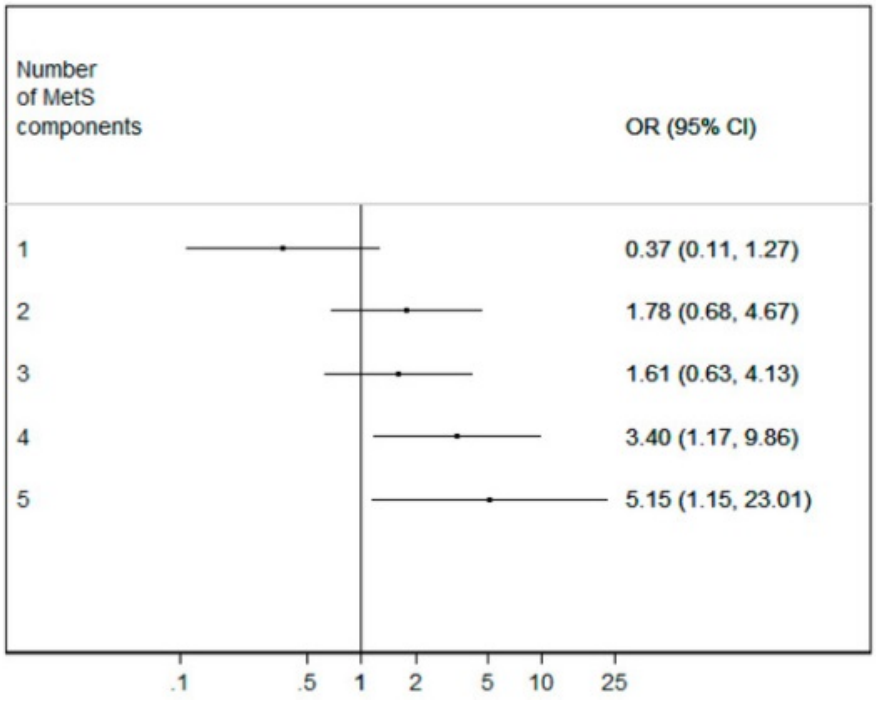

Figure 10. Tumor risk depending on the presence of different numbers of individual MetS components WD GEP-NEN (well-differentiated gastro-enteric-pancreatic neuroendocrine tumors), OR (odds ratio), CI (confidence interval), and MetS (Metabolic Syndrome).

No association was found between WD GEP-NEN and BMI categories (p = 0.851 for excess weight and p = 0.847 for obesity) or the presence of T2-DM (p = 0.608) even though IFG was significantly more frequent in patients than in controls (p = 0.013).

3.2.6 Discussion

Obesity and MetS are well established risk factors for several cancers even though whether there is a link between these conditions and the recent burden of GEP-NEN is yet to be confirmed. The aim of this study was to investigate whether there was an association between WD GEP-NEN and the anthropometric and

metabolic abnormalities that characterize MetS. Our results show that WD GEP-NEN are associated with MetS and some of the MetS individual components including elevated WC as surrogate for visceral obesity, fasting TG, and FPG. Moreover, the association was significantly increased if four or five individual MetS components were present. These findings also suggest WD GEP-NEN could also be associated with visceral obesity and severe IR despite the fact that no clear association with obesity grade or T2-DM was found. Therefore, this data proposes that poor metabolic health, characterized by visceral obesity with altered glucose and lipid metabolism, are the most likely risk determinants of WD GEP-NEN. Similar association profiles were also described for other types of cancers including colon and rectal cancer (Kim, Jung et al. 2018), prostate cancer (Chen, Deng et al. 2018), esophageal cancer (Liu, Cheng et al. 2018), and even thyroid cancer (Yin, He et al. 2018).

One of the main strengths of this study was enrolling a reasonably large patient sample with consistent data retrieval for what is considered a rare disease. All clinical and anthropometrical parameters were collected by the same researcher. Matching controls for age, gender, and the area of residence derived from the same background population ensured that these variables were similarly distributed in both groups. However, some limitations must be acknowledged. First, this was a single center-based case-control study. Additionally, due to the tertiary nature of our referral center, the PT removal and SA treatment initiation had already occurred when first observed at our institution in a considerable proportion of patients. In these circumstances, data was obtained retrospectively to reassure patient status before treatment. The sole exception was for FPI and FPG assessment that were performed while on SA to minimize the hyperglycemic effect of the treatment sampling that was made immediately before the next dose (Verges, Walter et al. 2014). Furthermore, as ongoing therapies were not subjected to match-control, the proportion of patients under BP or lipid lowering therapy was significantly higher in WD-GEP-NEN patients than in controls. This fact is unsurprising since subjects included in the control group were attended by general practitioners while patients with NEN were attended at tertiary centers where treatment intensification is more likely to occur. However, this dissimilarity between the groups should be interpreted into context because, according to the

established JIS criteria for MetS of the International Diabetes Federation Task Force on Epidemiology and Prevention, ongoing treatment for any of the individual parameters is considered equivalent to the positive individual criteria regardless of the glucose, lipid, or BP observed. Second, although the majority of patients under lipid lowering therapies, were already under treatment when first observed, these therapies were mainly statins (91.7%) that target mostly TC and LDL-c, which is less likely to interfere with TG and HDL-c levels and biased MetS syndrome individual criteria. Third, the fact that a larger percentage of patients with NEN were under anti-hypertensive drugs for a similar BP levels further suggests the dissimilarity between the MetS risk profile between the two groups. Additional potential confounding factors such as a family history of cancer, cigarette smoking, alcohol consumption, dietary habits, physical activity, occupation, and socioeconomic status were not evaluated.

GEP-NEN were traditionally considered rare tumors. This paradigm has been changing over the last four decades since a nearly seven-fold increase in GEP-NEN incidence was registered with a current age-adjusted incidence of 6.4 cases/100,000 inhabitants, which renders the ranking of the second most prevalent digestive neoplasia after colorectal cancer (Yao, Hassan et al. 2008, Oberg, Knigge et al. 2012, Dasari, Shen et al. 2017, Pavel and de Herder 2017). The reasons for the upsurge in GEP-NEN have been mostly attributed to an increase in incidental discovery by the widespread use of imaging techniques and improved medical skills, while the actual mechanisms leading to the recent burden have not attracted extensive investigation and remain largely unknown. Nonetheless, epidemiological trends analysis using national statistics from several countries suggest that, to be able to explain the difference in geographic and ethnic incidence patterns, both genetic and environmental factors must be involved in the natural history of NEN (Huguet, Grossman et al. 2017).

Obesity is known to be associated with cancer since the fourth decade of the 20th century (Tannenbaum 1940). More recently, mechanisms that link obesity and cancer were also established and particularly visceral adiposity was found to be linked with an increased risk of cancer independently of BMI (Dong, Zhou et al. 2017). Given the rarity and heterogeneity of GEP-NEN, epidemiological studies designed to investigate the association between metabolic RFs for the disease

are lacking. Although obesity is not yet an established RF for GEP-NEN, few studies demonstrated that BMI increases panNEN risk. A meta-analysis published in 2016 (Leoncini, Carioli et al. 2016) describes two case-control studies linking BMI and panNEN (Hassan, Phan et al. 2008, Halfdanarson, Bamlet et al. 2014) with a pool risk of 1.37 (95% CI 0.25 to 7.69, $p < 0.001$). The prevalence of incidental gastric NEN in obesity surgery candidates was found to be high (Al-Harbi, Shakir et al. 2013) and the occurrence of a panNEN co-secreting GLP-1 and glucagon in a patient previously submitted for gastric bypass surgery was also reported (Guimaraes, Rodrigues et al. 2015). Although our data does not support an association between overweight or obesity with WD GEP-NEN, visceral obesity as assessed by the WC criteria for MetS was associated with an increased risk for WD GEP-NENs. Few studies have addressed the putative association between glucose abnormalities with NENs and the majority refers to panNEN. Diabetes is a hallmark of some rare functioning (RF) GEP-NEN such as glucagonomas, vasoactive intestinal polypeptide secreting tumors (VIPomas), and somatostatinomas and is present in 70% of non-functioning panNEN (Vinik and Gonzales 2011). Moreover, hyperglycemia can also be a side effect of chemotherapy, SA, everolimus, and more recently PRRT (Verges, Walter et al. 2014). Our results show that not only patients with pancreatic NEN but also GI-NEN especially small bowel have a higher prevalence of MetS and glucose metabolism abnormalities. The present study points to a strong association between all sites WD GEP-NEN and IFG even before the initiation of treatments that can cause altered glucose homeostasis. This association was not exclusive of panNEN since it was also found in GI-NEN. No RF GEP-NEN characterized by hyperglycemia were included in this cohort. A strong association between diabetes and panNEN with an estimate effect of 2.76 (95% CI 1.65–4.64, $p = 0.090$) was formerly found in three case-control studies (Hassan, Phan et al. 2008, Capurso, Falconi et al. 2009, Halfdanarson, Bamlet et al. 2014). This effect was even higher in cases with recent onset diabetes (OR 12.80, 95%CI 2.47–66.42, $p = 0.135$) and insulin treated patients (OR 4.80, 95% CI 1.20–18.90). Two studies previously described the association between diabetes and tumors other than panNEN. In women with pre-existing T2-DM, gastric endocrine neoplasia (especially T1-GEN) and small bowel NEN were found to be increased seven-fold and two-fold, respectively (Hassan, Phan et al. 2008).

Increased prevalence of IGT in patients with serotonin (5-Hydroxitriptamin, 5-HT) secreting metastatic NEN when compared to non-secreting tumors was initially reported in 1975 (Feldman, Plonk et al. 1975). Moreover, a recent publication from Valente et al. concluded that non-recent diabetes was associated with an increased occurrence of panNEN especially in metastatic disease and an advanced grade (Valente, Hayes et al. 2017).

Our findings also support that there is an association of MetS with WD GEP-NEN. There is accumulating evidence that visceral obesity, IR, hyperinsulinemia, chronic inflammation, and T2-DM can lead to increased cell proliferation, apoptosis inhibition, angiogenesis, and impaired immunity (Jee, Kim et al. 2005, Font-Burgada, Sun et al. 2016). MetS is a cluster of RFs with a well-established association with CVD disease that was also demonstrated to be a modifiable RF for several cancers (Uzunlulu, Telci Caklili et al. 2016) such as breast cancer in postmenopausal women (HR 1.89, 95% CI 1.29–2.77) (Agnoli, Grioni et al. 2015). Two studies from South Korea concluded that there is an association between MetS and rectal NEN (r-NENs) (OR 1.768, 95% CI 1.071–2.918, p = 0.026) (Jung, Yun et al. 2014, Pyo, Hong et al. 2016).

In the present study, no significant differences in FPI and HOMA-IR were found between patients and controls. Nonetheless, the proportion of severe IR (HOMA-IR ≥ 5) was significantly higher in patients than in controls. Despite a large proportion of patients being under SA at the time of FPI and an FG determination (60%), no differences in MetS criteria, MetS individual components, FPI, HOMA-IR, and the proportion of insulin resistant and severe insulin resistant patients were found between patients under SA treatment or were untreated, which suggests that our findings were not influenced by SA (Table XIII). Our results also show that, although no differences were found in median TG levels between patients and controls, the proportion of GEP-NEN patients with TG ≥ 150 mg/dL was significantly higher than in controls (p = 0.011). Despite the fact that low HDL-c was identified as an independent RF for r-NEN in a South Korean cohort (OR 1.85, 95% CI 1.10–3.11, p = 0.021) (Jung, Yun et al. 2014, Pyo, Hong et al. 2016), the unexpected finding of lower TC and LDL-c levels as well as higher HDL-c levels in our patients' cohort compared to controls could be attributed to treatment intensification of patients with GEP-NEN when compared to the general

population since 37.2% of the patients vs. 9.9% of controls were under drug treatment for dyslipidemia. Previously, only hypercholesterolemia was found to be a RF for rectal GEP-NEN (OR 1.007, 95% CI 1.001–1.013; p = 0.016) in a single study (Pyo, Hong et al. 2016). This is in contrast with hypertension since no association was found between hypertension and WD GEP-NEN.

3.2.7 Conclusions

In conclusion, our findings show that WD GEP-NEN are associated with MetS, elevated WC, elevated FPG, elevated TG, and severe IR. These results are a breakthrough towards understanding the recent WD GEP-NEN “epidemic” since the association of the anthropometric, clinical, and biochemical abnormalities that characterize MetS or IR with these specific tumors, according to the primary location, the hormonal functional status, and grading or staging that had not been previously reported. Although requiring confirmation in larger scale studies, these novel findings could provide crucial insights toward the understanding of putative mechanisms leading to disease and prove important to establish targeted preventive and treatment interventions (Anand, Kunnumakkara et al. 2008) by addressing cancer as a metabolic disease (Seyfried and Shelton 2010).

3.3 Disseminated Well-Differentiated Gastro-Entero-Pancreatic Tumors Are Associated with Metabolic Syndrome

3.3.1 Abstract

The association of WD GEP-NEN with MetS, abdominal obesity, and fasting glucose abnormalities was recently described. The aim of this study was to evaluate whether the presence of MetS or any MetS individual component was influenced by GEP-NEN characteristics at diagnosis. A cohort of patients with WD GEP-NEN (*n* = 134), classified according to PT location (gastrointestinal or pancreatic), pathological grading (G1 (Ki67 ≤ 2%) and G2 (>3 ≤ 20%) (WHO 2010), disease extension (localized, loco-regional, and metastatic), and presence of hormonal secretion syndrome (functioning/non-functioning), was evaluated for the presence of MetS criteria. After adjustment for age and gender, the odds of having MetS was significantly higher for patients with WD GEP-NEN grade G1 (OR 4.35 95%CI 1.30–14.53) and disseminated disease (OR 4.52 95%CI 1.44–14.15). GEP-NEN PT location or secretory syndrome did not influence the risk for MetS. None of the tumor characteristics evaluated were associated with BMI, FPG category, or any of the individual MetS components. Patients with GEP-NEN and MetS depicted a higher risk of presenting a lower tumor grade and disseminated disease. The positive association between MetS and GEP-NEN characteristics further highlights the potential link between the two conditions.

3.3.2 Introdution

GEP-NEN were previously a rare entity before the 6.5-fold increase in incidence observed over the past four decades (Dasari, Shen et al. 2017). As a matter of fact, GEP-NENs are now the second most frequent digestive tumors after colorectal adenocarcinoma (Yao, Hassan et al. 2008). The reasons underlying the exponential increase in the incidence of sporadic GEP-NEN remain largely unknown, even though significant advances toward understanding the genetics and molecular mechanisms associated with GEP-NEN biology were made (Zhang, Francois et al. 2013). One of the most remarkable achievements of oncology in the 21st century was the finding that most cancers could be preventable diseases (Anand, Kunnumakkara et al. 2008). The association of environmental factors with tumor development, disease recurrence, and mortality risks were demonstrated by a large number of studies for several different types of cancers (Demark-Wahnefried, Platz et al. 2012, Flegal, Kit et al. 2013, Arnold, Pandeya et al. 2015, Islami, Goding Sauer et al. 2018). In particular, obesity, MetS, and T2-DM, which are also experiencing an exponential rise worldwide, have been implicated as RFs for cancer incidence and disease recurrence (Esposito, Chiodini et al. 2012, You, Liu et al. 2015). Despite the available evidence that those metabolic conditions are RFs for several different tumor types, the amount of data available concerning GEP-NEN is more limited. The association between WD GEP-NEN with MetS and some of the MetS individual components, namely abdominal obesity and abnormal FPG, was recently described by our group (Santos, Santos et al. 2018).

3.3.3 Hypothesis And Aims

The aim of the current study was to evaluate whether the presence of MetS and individual MetS components at the time of WD GEP-NEN diagnosis was associated with any specific tumor characteristics, such as grading, staging, PT location, or hormonal hypersecretion, that were likely to influence the tumor biological behavior and disease prognosis.

3.3.4 Experimental Section

Patients with confirmed WD GEP-NEN were recruited from the Endocrine Tumors Clinic of a single large tertiary referral center for oncologic diseases, IPOFG, Porto. The inclusion criteria included having a confirmed diagnosis of WD GEP-NEN by histopathology and/or ^{68}Ga-PET-SSR. Patients excluded from the study were those who were younger than 18 years old when first diagnosed, as well as those harboring familial GEP-NEN, NEC, and/or a T1-GEN, as these tumors are recognized as having a distinctive and well-established etiology and biological behavior (Heetfeld, Chougnet et al. 2015).

From the patients with confirmed WD GEP-NEN that consented to participate in the study (*n* = 159), those who did not fulfill the inclusion criteria or had insufficient data for analysis were excluded, while the remaining eligible patients were included in the study for statistical analysis (*n* = 136). Tumors were classified according to PT location: GI-NEN or panNEN; functioning or non-functioning (F or non-F); pathological WHO grading into G1 (<2 mitotic count; Ki-67 index ≤ 2) and G2 (2–20 mitotic count; Ki-67 index 3–20) and disease extension (localized, loco-regional, and disseminated) (O'Toole, Kianmanesh et al. 2016). Disease extension was categorized as localized, locoregional, or disseminated, to enable the grouping of WD GEP-NEN, since ENETS staging categories, depending on the PT location, diverge. Patients with insufficient data to allow for grading were classified as WD GEP-NEN if found to express somatostatin receptors on ^{68}Ga-PET-SSTR (*n* = 6). Patients with WD GEP-NEN metastatic tumors and carcinoid syndrome without any visible pancreatic or thoracic lesions on imaging studies were assumed as midgut PT (*n* = 2). No insulinoma or rare functional pancreatic NEN presenting with hyperglycemia, such as glucagonoma, VIPoma, or somatostatinoma, were included in this series (O'Toole, Salazar et al. 2006).

Patients with WD GEP-NEN were assessed for BMI class (Borrell and Samuel 2014), FPG category (American Diabetes 2010), and the presence or absence of MetS diagnostic criteria or any individual MetS component (Alberti, Eckel et al. 2009).

Data for analysis were collected during face-to-face patient interviews, to assess past medical history of T2-DM, hypertension, dyslipidemia, ongoing medications, and family history of T2-DM. Anthropometric parameters, such as height, weight, WC, and BP were measured during the study visit. Additionally, biochemical data, including FPG and lipid profile, were evaluated after blood sampling in our institution for treatment-naïve patients, or retrospectively through data-files recollection of parameters before initiation of any treatment intervention at the referring healthcare institutions, whenever the patient was already under pharmacological treatment when first observed at our center.

Patients were classified into three categories according to BMI: normal weight (BMI < 25 kg/m^2), overweight (BMI 25–29.9 kg/m^2), or obese (BMI ≥ 30 kg/m^2) (Borrell and Samuel 2014). They were also classified according to FPG levels: normoglycemic (NG; FPG < 100 mg/dL), impaired fasting glucose (IFG; FPG ≥ 100 < 126 mg/dL), or T2-DM (T2-DM; FPG ≥ 126 mg/dL) (American Diabetes 2010). MetS was classified according to the JIS of International Diabetes Federation Task Force on Epidemiology and Prevention (IDFTFEP) /NHLBI (National Heart, Lung, and Blood Institute)/AHA (American Heart Association)/WHF/IAS /IASO (International Association for the Study of Obesity) criteria (Alberti, Eckel et al. 2009): WC ≥ 88 cm (female) or 102 cm (male); systolic BP ≥ 130 or diastolic BP ≥ 85 mmHg or previous history of high BP or under BP-lowering medication; HDL-c < 40 mg/dL (male) or < 50 mg/dL (female) or drug treatment to reduce HDL-c; TG ≥150 mg/dL or under triglyceride-lowering drugs; FPG ≥ 100 mg/dL or ongoing treatment with glucose-lowering drugs.

This study was approved by the National Data Protection Committee (CNPD /4906/2015) and Institutional Ethics Review Board (IPOP/366/2013). All participants provided informed consent prior to study enrolment.

Statistical analysis was performed using IBM SPSS Statistics version 24.0 (IBM, New York, USA). Categorical and continuous variables were summarized using descriptive statistics (frequencies for categorical; mean/standard deviation or median/interquartile range for continuous, as appropriate). Proportions were compared using the Chi-square or Fisher's exact test, as appropriate. Means were compared using Student's *t* test or ANOVA, while medians were compared

using the Mann–Whitney or Kruskal–Wallis tests. A backward stepwise (Wald) method was used to obtain a multivariable logistic regression model, using the patient and tumor characteristics (sex, age at diagnosis, tumor primary site, grading, stage, and clinical hypersecretion syndrome). A level of significance of 0.05 was adopted.

3.3.5 Results

The cohort of patients with WD GEP-NEN (n = 134) was divided into two groups, according to baseline characteristics and considering the absence (n = 57) or presence of MetS (n = 77) at the time of tumor diagnosis (Table XVI). Patients in the group with MetS were predominantly male (p = 0.014), older (p < 0.001), and had a higher BMI at diagnosis (p < 0.001). When comparing the two patient groups, there was a homogeneous distribution in terms of PT location (p = 0.652), presence of hormonal secretion syndrome (p = 0.187), and metastatic disease (p = 0.104). Grade 1 (G1) tumors were found to be more frequent in the MetS group; although, the difference was not statistically significant (p = 0.076) (Table XVI).

Table XVI. General patient and WD GEP-NEN characteristics (n=136) in the two patient groups according to the presence of Metabolic Syndrome diagnostic criteria

WD GEP-NEN	**Without MetS (*n* = 57)**	**With MetS (*n* = 77)**	***p***
Gender-*n* (%)	21 (36.8) M/36 (63.2) F	46 (59.7) M/31 (40.3) F	0.009
Age-Mean (min.-max.)	57.2 (30–78) y	65.9 (42–85) y	<0.001
Age at Diagnosis (min.-max.)	53.9 (29–78) y	62.4 (38–85) y	<0.001
Weight (kg)-Mean ± SD	65.7 ± 11.4	76.4 ± 12.8	<0.001
BMI (kg/m^2)-Median (IQR)	24.3 (4.05)	27.8 (5.47)	<0.001
WC (cm)-Mean ± SD	87.5 ± 10.6	99.3 (10.5)	<0.001
SBP (mmHg)-Mean ± SD	127.8 ± 14.6	140.7 ± 22.1	<0.001
DBP (mmHg)-Mean ± SD	72.9 ± 10.2	75.2 ± 12.4	0.262
HDL-c (mg/dL)-Mean ± SD	55.5 ± 13.2	46.6 ± 11.0	<0.001
Triglycerides (mg/dL)-Median IQR)	99.0 (13.0)	137.0 (83.5)	<0.001
FPG (mg/dL)-Median (IQR)	92.0 (13.0)	109.0 (18.5)	<0.001
Primary Tumor Location (*n* = 131)			0.652
GI-NET	43 (76.8)	55 (73.3)	
pNET	13 (23.2)	20 (26.7)	
Hormonal Syndrome (*n* = 119)			0.187
Functioning *	17 (32.1)	36 (67.9)	
Non-Functioning	29 (43.9)	37 (56.1)	
2010 WHO Gradinge (n=127) #			0.076
Grade 1	34 (61.8)	55 (76.4)	
Grade 2	21 (38.2)	17 (23.6)	
Staging (*n* = 122)			0.104
Localized Disease	24 (46.2)	22 (31.4)	
Locoregional Disease	10 (59.2)	10 (14.3)	
Metastatic Disease	18 (34.6)	38 (54.3)	
Extra-Hepatic Metastatic Disease ♀	5 (26.3)	8 (21.1)	0.448
Neuroendocrine Tumors pt. Treatments (*n* = 134)			
Surgery *-*n* (%)			
Liver Ablative Therapies–*n* (%)	10 (17.5)	20 (26.0)	0.298
Somatostatin Analogues–*n* (%)	35 (45.5)	42 (54.5)	0.383
Target Therapies–*n* (%)	-	-	-
PRRT–*n* (%)	5(8.8)	4 (5.2)	0.495
Chemotherapy–*n* (%)	1(1.8)	1 (1.3)	1.000

*WD GEP-NENs (well-differentiated gastro-entero-pancreatic neuroendocrine neoplasia); MetS (metabolic syndrome); BMI (body mass index); WC (waist circumference); SBP (systolic blood pressure); DBP (diastolic blood pressure); FPG (fasting plasma glucose); GI-NEN (gastrointestinal neuroendocrine neoplasia); panNEN (pancreatic neuroendocrine neoplasia); WHO (World Health Organization); ENETS (European Neuroendocrine Tumor Society); PRRT (peptide receptor radionuclide therapy) * 49/119 (41.8%) patients with carcinoid syndrome (33 patients with MetS and 13 patients without MetS) and 2/119 (1.7%) patients with sporadic gastrinoma (100% with MetS)). # WHO 2010 Grade was used since 2013 and was the date of first patient enrolment, ♀ 3/13 bone metastasis; 8/13 peritoneal implants and 2/13 other locations.*

The odds of patients with WD GEP-NEN having MetS was significantly higher in males (p = 0.009) and increased with age (p < 0.001) (Figure 11).

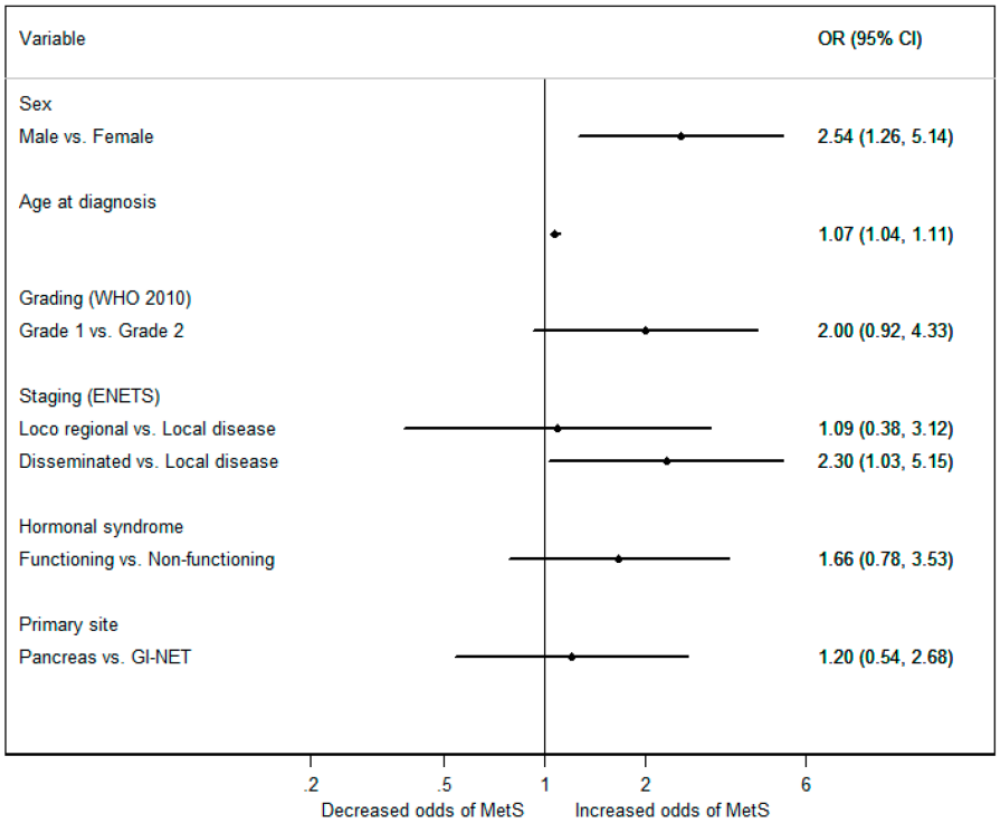

Figure 11. Odds ratios (ORs) and 95% confidence intervals (CIs) for the occurrence of Metabolic Syndrome according to the characteristics of patients with WD GEP-NEN, using a univariate logistic regression.

After adjusting for age and gender, the positive association between disseminated disease and MetS persisted, with patients with metastatic disease depicting odds of having MetS over four times greater than patients with localized disease (OR 4.52 95%CI 1.44–14.15; p = 0.010). In addition, G1 grade was found to be significantly associated with MetS (NET G2 vs. G1; (OR 4.35 95%CI 1.30–14.53; p = 0.018), while the PT location or hormonal secretory status of GEP-NEN did not influence the risk of MetS (Fig. 12).

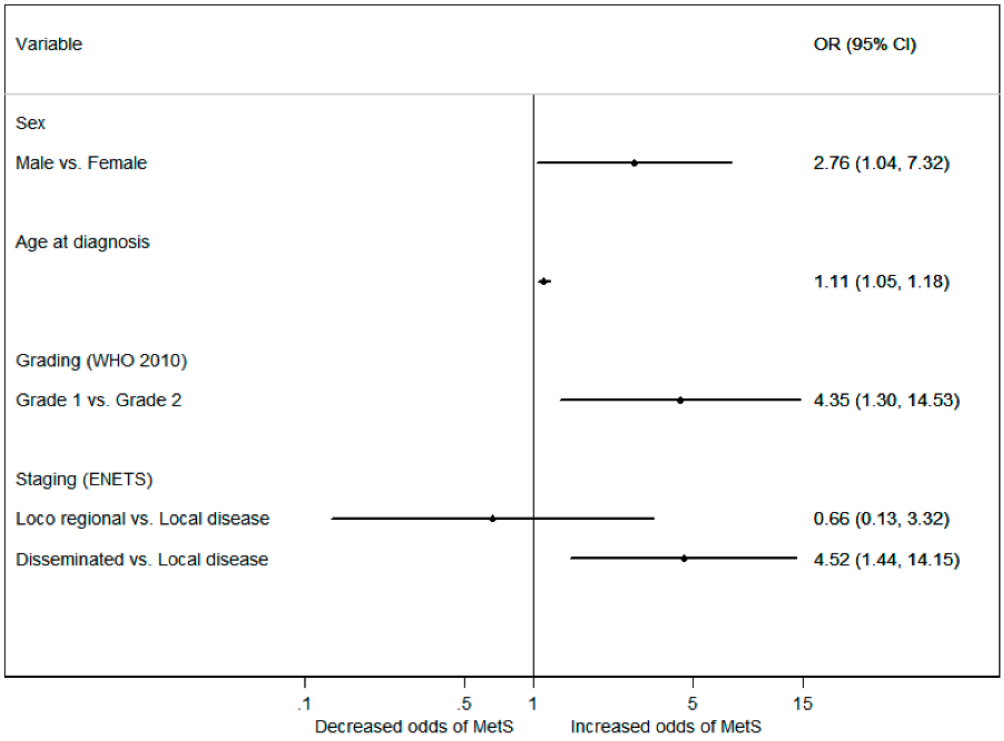

Figure 12. Odds ratios (ORs) and 95% confidence intervals (CIs) for the occurrence of Metabolic Syndrome according to the characteristics of patients with WD GEP-NEN, using a multivariate logistic regression.

No significant association was found between WD GEP-NEN`s primary tumor location, presence of hormonal secretion syndrome, tumor grading or disease extension and the presence of any of the individual MetS components at diagnosis (Table XVII).

Table XVII. Presence of Metabolic Syndrome individual components in patients with WD GEP-NEN according to tumor characteristics (n=136)

	Abdominal Obesity		Hypertension		Low HDL-c		High TG		High FPG	
	n (%)	*p*	*n* (%)	*p*	*n* (%)	*p*	*n* (%)	*p*	*n* (%)	*p*
Primary Tumor Location (*n* = 133)		0.536		0.084		0.803		0.384		0.194
GI-NEN	47(51.6)		69 (70.4)		51 (52.0)		35 (35.7)		52 (53.1)	
panNEN	18 (58.1)		19 (54.3)		18 (54.5)		15 (44.1)		21 (60.0)	
Hormonal Syndrome (*n* = 121)		0.430		0.268		0.430		0.507		0.673
Functioning	28 (58.3)		39 (72.2)		34 (63.0)		22 (40.7)		31 (57.4)	
Non-Functioning	32 (50.8)		42 (62.7)		30 (45.5)		23 (34.8)		41 (61.2)	
WHO Grade (*n* = 129)		0.648		0.178		0.601		0.978		0.515
Grade 1	41 (48.8)		62 (69.7)		49 (55.1)		34 (38.2)		50 (56.2)	
Grade 2	18 (52.9)		23 (57.5)		19 (50.0)		15 (38.59		20 (50.2)	
ENETS Staging (*n* = 124)		0.633		0.677		0.092		0.336		0.194
Localized Disease	24 (46.2)		30 (65.2)		20 (44.4)		16 (34.8)		24 (52.2)	
Locoregional Disease	10 (59.2)		13 (65.0)		11 (55.0)		7 (35.0)		8 (40.0)	
Metastatic Disease	18 (34.6)		40 (69.0)		35 861.4)		25 (43.9)		37 (63.8)	

WD GEP-NENs (well-differentiated gastro-entero-pancreatic neuroendocrine neoplasia); MetS: metabolic syndrome;
GI-NEN (gastrointestinal neuroendocrine neoplasia); panNEN: pancreatic neuroendocrine neoplasia;
WHO (World Health Organization); ENETS (European Neuroendocrine Tumor Society).

Also, we didn`t find any significant association between WD GEP-NEN characteristics and BMI or FPG classification (Table XVIII).

Table XVIII. Association of WD GEP-NEN characteristics with the BMI grade and fasting plasma glucose (FPG) classification at diagnosis

WD GEP-NENs	BMI Grade				FPG Classification			
	Normal	Overweight	Obesity	*P*	Normal	AFPG	T2DM	*p*
Primary Tumor Location (*n* = 132)				0.187				0.326
GI-NEN	31 (64.6)	42 (76.4)	24 (82.8)		59 (74.7)	22 (81.5)	17 (63.0)	
panNEN	17 (35.4)	13 (26.3)	5 (17.2)		20 (25.3)	5 (18.5)	10 (37.0)	
Hormonal Syndrome (*n* = 120)				0.281				0.281
Functioning	28 (63.6)	23 (46.9)	15 (55.6)		36 (53.7)	11 (45.8)	20 (66.7)	
Non-Functioning	16 (36.4)	26 (53.1)	12 (44.4)		31 (46.3)	13 (54.2)	10 (33.3)	
WHO Grade (*n* = 129)				0.622				0.698
Grade 1	17 (36.2)	16 (29.6)	7 (25.9)		20 (34.2)	7 (26.9)	7 (25.9)	
Grade 2	30 (63.8)	38 (70.4)	20 (74.1)		50 (65.8)	19 (73.1)	20 (74.1)	
ENETS Staging (*n* = 124)				0.234				0.251
Localized Disease	17 (39.5)	18 (33.3)	10 (38.5)		29 (39.7)	9 (34.6)	8 (32.0)	
Locoregional Disease	9 (20.9)	5 (9.3)	6 (23.1)		14 (19.2)	1 (3.8)	5 (20.0)	
Metastatic Disease	17 (	31 (57.4)	10 (38.5)		30 (41.1)	16 (61.5)	12 (48.0)	

WD GEP-NEN (well-differentiated gastro-entero-pancreatic neuroendocrine neoplasia); BMI (body mass index);
FPG (fasting plasma glucose); MetS (metabolic syndrome); GI-NEN: gastrointestinal neuroendocrine neoplasia;
panNEN (pancreatic neuroendocrine neoplasia); WHO (World Health Organization);
ENETS (European Neuroendocrine Tumor Society).

3.3.6 Discussion

GEP-NEN are a group of heterogeneous neoplasms that may present considerable differences in what concerns PT location, pattern of hormone secretion, proliferative behavior, and disease extension at diagnosis. Obesity, MetS, and T2-DM were recognized as RFs for several cancers (Esposito, Chiodini et al. 2012, Arnold, Pandeya et al. 2015, Pearson-Stuttard, Zhou et al. 2018). These include esophageal, pancreatic, colorectal, endometrial, kidney, and breast cancer in post-menopausal women (Suh and Kim 2011, Rahib, Smith et al. 2014, Arnold, Pandeya et al. 2015, Uzunlulu, Telci Caklili et al. 2016, Heckman-Stoddard, DeCensi et al. 2017, Brown, Rumgay et al. 2018, Pearson-Stuttard, Zhou et al. 2018, Barberio, Alareeki et al. 2019). However, whether any of these metabolic conditions are also RFs for GEP-NEN or are able to negatively influence disease behavior is yet to be fully established. Notwithstanding, our group has shown in a case-control study that WD GEP-NEN are associated with visceral obesity, elevated FPG, and MetS (Santos, Santos et al. 2018). Given these prior findings, our current aim was to investigate whether there were any further associations between the pathological features of WD GEP-NEN and the anthropometric and clinical parameters that characterize MetS.

The incidence of GEP-NENs increased over the last four decades, disclosing a current prevalence of 6.98 cases/100,000 inhabitants (Yao, Hassan et al. 2008, Dasari, Shen et al. 2017). The upsurge in GEP-NEN was initially attributed to improved medical skills, which led to an increased rate of incidental diagnosis by the widespread use of imaging techniques, while the search for other possible mechanisms underlying the unprecedented disease burden did not attract extensive investigation. Still, epidemiological data derived from several national registries suggest that both genetic and environmental factors must be involved in the phenomenon, explaining the ethnic and geographical differences observed in GEP-NEN patterns (Boyar Cetinkaya, Aagnes et al. 2017). Nevertheless, most studies that were aimed at unravelling the biology of GEP-NEN focused primarily on tumor genetics or molecular pathways underlying intrinsic pathological features (Capurso, Falconi et al. 2009, Zhang, Francois et al. 2013, Zhang, Li et

al. 2019), while the potential contribution of environmental factors was mostly neglected. Indeed, only a small number of retrospective studies have addressed the potential relationship between obesity, MetS, or T2-DM and GEP-NEN (Hassan, Phan et al. 2008, Capurso, Falconi et al. 2009, Halfdanarson, Bamlet et al. 2014), and the rare studies available were predominantly dedicated to panNEN only (Valente, Hayes et al. 2017, Gallo, Ruggeri et al. 2018). In 2016, the largest subset meta-analysis ever performed disclosed BMI and T2-DM, in addition to family history of cancer, as unpredicted RFs for stomach, pancreas, and small-intestine GEP-NEN (Leoncini, Carioli et al. 2016). Furthermore, visceral obesity, high plasma TG, abnormal FPG, and MetS were found to be associated with an increased risk of WD GEP-NEN in a case-control study performed by our group (Santos, Santos et al. 2018). Previously, MetS was identified only as a RF for a subgroup of rectal WD GEP-NEN by two independent studies conducted in South Korea (Jung, Yun et al. 2014, Pyo, Hong et al. 2016).

The core pathological feature that characterizes MetS is hyperinsulinism. In turn, hyperinsulinism leads to the subsequent activation of the insulin-IGF1 axis that has been theoretically proposed to support the relevance of MetS for WD GEP-NEN biology (Djiogue, Nwabo Kamdje et al. 2013). Consequently, the use of insulin-sensitizing agents able to mitigate hyperinsulinism, such as metformin, for the prevention and treatment of cancer was also suggested. Indeed, the potential benefits of metformin as an anticancer drug are supported not only by several in vitro and in vivo experimental studies (Rizos and Elisaf 2013), but also by human data derived from epidemiological studies and prospective clinical trials (Zi, Zi et al. 2018). Nevertheless, the proposed mechanisms responsible for the anticancer effects of metformin are not only limited to the improvement of insulin sensitivity, decreased hyperinsulinism, and the inhibition of the insulin-IGF1 axis, but also other potential direct actions, such as inhibiting the MAPK and /Akt/PI3K/mTor pathway and enhancing $CD8^+$ T cells, which are key players in mediating immunity to tumors, for immune-mediator anticancer effects (Yu, Mao et al. 2017). Indeed, the inhibition of the Akt/PI3K/ mTor pathway is a well-known target for NEN therapy, with everolimus being approved for the treatment of metastatic unresectable WD panNEN (Yao, Shah et al. 2011).

Our study aimed to evaluate the association of the four main characteristics of WD GEP-NEN, namely PT location, presence of hypersecretion syndrome, WHO grade and stage, with the occurrence of MetS. We were able to demonstrate, for the first time, that patients with WD GEP-NEN and MetS, independent of age or gender, are more likely to have lower-grade tumors or present advanced-stage disease at diagnosis. In fact, despite the fact that patients with MetS were more likely to be older, in parallel to what is observed in the general background population, this parameter was not shown to influence WD GEP-NEN characteristics (Raposo, Severo et al. 2017). Moreover, neither the WD primary tumor location of GEP-NEN nor the presence of hormonal secretion syndrome was associated with MetS or any of the individual components of MetS. Furthermore, although metabolic alterations are usually associated with functioning and non-functioning panNEN, in this cohort, functioning GI-NEN with carcinoid syndrome were also shown to be associated with MetS in 41.8% of the cases. In fact, a considerable number of subjects in our cohort had small-intestinal WD GEP-NEN that, despite presenting small-size PTs, were often metastatic at diagnosis. Of particular note is the fact that more than half of the patients with metastatic disease also had MetS features. This observation raises the need to investigate the impact of WD GEP-NEN on MetS, as the mechanistic reasons for this observation are not entirely clear and thus warrant further investigation. Notwithstanding the widespread dissemination of the disease, patients with GEP-NEN usually preserve an overall very good health status, with rare cases of cancer cachexia, which is particularly notorious in patients with GI-NEN, as confirmed by the nearly two-thirds of patients with an overweight or obesity BMI grade.

One of the main strengths of this study is that it enrolled a reasonably large patient cohort for what is considered a relatively rare disease, along with consistent data retrieval, since all clinical and anthropometrical parameters were assessed by a single clinical researcher. However, some limitations must also be acknowledged. First, this study was conducted in a single center; therefore, and despite the sample size, these results require further validation, ideally in multicenter prospective studies. In addition, since our study was conducted in an end-of-line tertiary center, a small proportion of patients with WD GEP-NEN were

already under pharmacological treatment for the disease when referred and first evaluated at our center. For these cases, clinical parameters were obtained retrospectively to minimize any possible bias for statistical analysis concerning data before treatment initiation. Last, but not least, other potential confounding factors, such as family history of cancer, cigarette smoking, alcohol consumption, dietary habits, physical activity, occupation, and socioeconomic status, were not evaluated in this study; therefore, we are unable to estimate whether these could have had any impact on the study results.

Overall, our data emphasize the unmet need to further explore the mechanisms underlying the association of obesity, abdominal obesity, and the metabolic abnormalities that characterize MetS with GEP-NEN, as such an exploration could not only improve the knowledge of the causes for the recently increased burden of these tumors, but it could also open a field of work that might lead to the disclosure of novel and more effective preventive and treatment avenues, as already described for other types of cancer.

In conclusion, this study demonstrates, for the first time, a positive association of MetS with WD GEP-NEN disease extension and tumor grade. Our results demonstrate that patients with WD GEP-NEN and MetS are more likely to have tumors with better differentiation and disseminated disease at diagnosis, independent of PT location and hormonal status. Our findings suggest that further research on the mechanisms underlying the metabolic abnormalities associated with WD GEP-NEN is warranted, as these underlying mechanisms could potentially harbor the keys for novel and more effective preventive and treatment interventions.

3.4 Higher IL-6 Peri-Tumoral Expression is Associated with Gastro-Intestinal Neuroendocrine Neoplasia Progression

3.4.1 Abstract

An association of WD GEP-NEN with MetS was recently described. Yet no molecular mechanisms linking the two conditions are known. This study's aim was to identify putative molecular signatures linking WD GEP NEN and MetS to gain further insight into potential mechanisms for this association. Patients with WD GEP NEN (n=39), panNEN and GI-NEN, were clinically evaluated for presence of MetS. WD GEP NEN immunohistochemistry staining for FOXM1, IGF1R, Ki-67 and IL-6 was performed and quantified by computerised morphometric analysis. FOXM1, Ki-67, IGF1R or IL-6 expression in WD GEP NEN was not influenced by the presence of MetS. IL-6 peritumoral expression was higher in GI-NEN of patients with low HDL-c (0.018±0.005% vs 0.030±0.005%, p=0.02). In GI-NEN, a higher IL-6 expression was also associated with disease progression (0.026±0.004% vs 0.016±0.002%, p=0.03).

In WD GEP-NEN, MetS did not influence FOXM1, IGF1R and IL-6 expression. In GI-NEN, IL-6 expression was influenced by the MetS feature low HDL-c, and positively associated with disease progression. These data suggest that local and systemic inflammatory status can potentially modulate GI-NEN behaviour.

3.4.2 Introduction

GEP-NEN comprise a group of rare and heterogeneous neoplasms that emerge from enterochromaffin epithelial cells of the diffuse endocrine system sparsely throughout the gastrointestinal tract and pancreas (Starker and Carling 2009, Sahani, Bonaffini et al. 2013). GEP NETs were previously considered rare neoplastic diseases. However, epidemiological data have shown an increase in

the incidence and prevalence of GEP-NEN over the last decades, which was attributed to increased disease awareness and diagnosis driven by the technical improvements observed in imaging and endoscopic techniques employed (Fraenkel, Kim et al. 2014, Dasari, Shen et al. 2017). Indeed, GEP-NEN are currently the second most common gastrointestinal malignancy after colorectal cancer (Yao, Hassan et al. 2008).

The prevalence of obesity, metabolic syndrome (MetS) and T2-DM is also escalating worldwide (Reynolds and He 2005, Forouhi and Wareham 2014, Friedrich 2017). Indeed, links between MetS or MetS individual components and cancer were recently demonstrated for several different malignancies, including endometrial cancer, colorectal cancer and hepatocarcinoma (Braun, Bitton-Worms et al. 2011, Arcidiacono, Iiritano et al. 2012, Esposito, Chiodini et al. 2012). The relationship between MetS and GEP-NEN is not as well established, although our group has recently reported that MetS and some MetS individual components, including visceral obesity, dyslipidaemia and high fasting glucose were associated with an increased risk for WD GEP-NEN (Santos, Santos et al. 2018). IR is known to play a key role in the aetiology of MetS (Reynolds and He 2005, Asrih and Jornayvaz 2015). IR states are responsible for an adaptive increase in circulating insulin levels as a counter regulatory response to overcome the resistance. The molecular links between IR and cancer are far from being entirely disclosed. Among the potential candidates are tyrosine kinase receptors (TKRs) signalling pathways, since these are most frequently found to be altered in human cancers. Indeed, insulin can activate MAPK and PI3K/AKT/mTOR pathways through IGF1R signaling (Djiogue, Nwabo Kamdje et al. 2013, Sever and Brugge 2015). In addition, IGF1R is highly expressed in WD GEP-NEN and is considered a potential molecular target for a variety of cancer therapies (Raymond, Hobday et al. 2011, Briest and Grabowski 2014, Briest, Berg et al. 2015, Dasari, Phan et al. 2015). FOXM1 is an essential transcription factor that cross-talks with MAPK and PI3K/AKT/mTOR pathways activation and consequently plays a major role in cell differentiation, cell cycle progression, cell proliferation and tumourigenesis among other biological processes (Wang, Ahmad et al. 2010). FOXM1 overexpression is observed in the majority of human solid cancers, including in WD GEP-NEN (Wierstra 2013, Gartel 2017).

Besides that, obesity and MetS are often accompanied by a systemic chronic inflammatory state, in which proinflammatory cytokines such as IL-6 are involved (Monteiro and Azevedo 2010, Tanaka, Narazaki et al. 2014). Therefore, IL-6 is often expressed in tumor surrounding tissues while being responsible for shaping the tumor microenvironment (Fisher, Appenheimer et al. 2014).

3.4.3 Hypothesis and Aims

In order to gain further insight into potential mechanisms underlying the association of MetS and WD GEP-NEN, the purpose of this study was to evaluate the influence of MetS criteria and MetS individual components in the expression of different molecular markers that participate in inflammatory (IL-6) and WD GEP-NEN metabolic pathways (IGF1R and FOXM1).

3.4.4 Material and Methods

3.4.4.1. Patients

Patients diagnosed with WD GEP-NEN (n=39) attending a single tertiary referral centre for endocrine tumors were enrolled in this study. Patients were divided into two main groups according to the location of the primary tumor: GI-NEN (n=29), and panNEN (n=10) (Table XIX). Presence of MetS was established according to JIS of NHLBI/AHA/WHF/IAS/IASO criteria (Alberti, Eckel et al. 2009) which defines the diagnosis of MetS in the presence of at least three of the five RFs: FPG ≥ 100 mg/dL or ongoing glucose-lowering drug treatments; WC ≥ 88 cm (female) or ≥102 cm (male); SBP ≥ 130 mm Hg or DBP ≥ 85 mm Hg or under BP lowering medications; HDL-c <40 mg/dL (male) or <50 mg/dL (female) or drug treatment for reduced HDL-c; TG ≥ 150 mg/dL or under triglyceride lowering drugs (Table II). All clinical parameters were assessed before surgical intervention for tumor removal.

Table XIX. Patients clinical features and tumor characteristics

	panNEN (n=10)	GI-NEN (n=29)
Patients clinical features		
Sex		
Female (%)	6 (60.00%)	12 (41.38%)
Male (%)	4 (40.00%)	17 (58.62%)
Median Age, years (range)	57 (29-75)	64 (41-81)
Metabolic Syndrome		
Absent (%)	4 (40.00%)	9 (31.03%)
Present[1] (%)	5 (50.00%)	20 (68.97%)
BMI	26.65 ± 1.76	27.58 ± 0.66
Normal weight (%)	5 (50.00%)	8 (27.59%)
Overweight (%)	2 (20.00%)	12 (41.38%)
Obese (%)	3 (30.00%)	9 (31.03%)
Waist circumference (cm)	93.73 ± 3.18	96.75 ± 2.87
< 102cm in males or <88cm in females (%)	4 (40.00%)	12 (41.38%)
≥ 102cm in males or ≥ 88cm in females (%)	5 (50.00%)	16 (55.17%)
Fasting plasma glucose (mg/dL)	103.10 ± 3.64	105.97 ± 4.20
< 100 mg/dL (%)	5 (50.00%)	9 (31.03%)
≥ 100 mg/dL (%)	5 (50.00%)	20 (68.97%)
Type 2 Diabetes Mellitus		
Absent (%)	7 (70.00%)	21 (72.41%)
Present (%)	2 (20.00%)	8 (27.59%)
HDL (mg/dL)	53.70 ± 4.50	48.48 ± 2.68
≥ 40 in males or ≥ 50 mg/dL in females (%)	5 (50.00%)	12 (41.38%)
< 40 in males or < 50 mg/dL in females (%)	5 (50.00%)	17 (58.62%)
Triglycerides (mg/dL)	122.70 ± 12.87	153.69 ± 15.31
<150 mg/dL(%)	5 (50.00%)	17 (58.62%)
≥ 150 mg/dL (%)	5 (50.00%)	12 (41.38%)
SBP (mm Hg)	136.60 ± 4.41	131.76 ± 2.84
DBP (mm Hg)	77.10 ± 2.86	72.83 ± 2.12
SBP <130 mmHg or DBP <85 mmHg (%)	4 (40.00%)	9 (31.03%)
SBP ≥ 130 mmHg or DBP ≥ 85 mm Hg (%)	6 (60.00%)	20 (68.97%)
Somatostatin analogues treatment		
No (%)	7 (70.00%)	8 (27.59%)
Yes (%)	3 (30.00%)	21 (72.41%)
Duration (months)	78.00 ± 24.74	67.05 ± 6.29
Tumors characteristics		
WHO grade		
G1 (%)	6 (60.00%)	24 (82.76%)
G2 (%)	4 (40.00%)	5 (17.24%)
Staging		
Local disease (%)	6 (60.00%)	5 (17.24%)
Loco regional disease (%)	1 (10.00%)	2 (13.79%)
Disseminated disease (%)	3 (30.00%)	20 (60.97%)
Functionality		
Functioning (%)	2 (20.00%)	22 (75.86%)
Non-functioning (%)	7 (70.00%)	7 (24.14%)
Disease status		
Stable disease (%)	2 (20.00%)	10 (34.48%)
Disease Free (%)	3 (30.00%)	6 (20.69%)
Disease Progression [(1)]	5 (50.00%)	13 (44.83%)
Progression-free survival (months)	83.44 ± 14.22	57.65 ± 5.98
Overall survival (months)	94.27 ± 14.42	77.27 ± 6.28

MetS – Metabolic syndrome; BMI – Body mass index; SBP- Systolic Blood Pressure; DBP- Diastolic Blood Pressure; HDL- High-density lipoprotein; panNET – pancreatic neuroendocrine tumors; GI-NET – gastrointestinal neuroendocrine tumors; (1) Defined as tumor progression after the first treatment. The divergence between number of patients and sum of studied parameters translates missing data.

3.4.4.2. Immunohistochemistry

Tissue sections (3 mm thick) were dewaxed in xylene and progressively hydrated in a decreasing scale of alcohols (100%, 95% and 70%) until water. Antigen retrieval was performed by incubation in a 10 mM citrate buffer (pH 6.0) with Tween 20 at 0.05% in a microwave at 900 W for 20 min for interleukin 6 (IL-6); by incubation in a 10 mM citrate buffer (pH 6.0) with Tween 20 at 0.05%, in a microwave at 900 W for 25 min after boiling for Forkhead box protein M1 (FOXM1); and by incubation in a 10 mM citrate buffer (pH 6.0) with Tween 20 at 0.05%, in a pressure cooker for 4 min after boiling for insulin growth factor 1 receptor (IGF1R). All the washes required throughout the process were performed in a phosphate buffered saline solution with Tween 20 at 0.05% (pH 7.4). Endogenous peroxidase was inhibited with the incubation of the sections in a solution of hydrogen peroxide and methanol at 3% for 20 min. Incubation with the respective primary antibody, was performed overnight at 4ºC: anti-IGF1R (1:100, ab39675; Abcam, UK), anti-FOXM1 (1:500, sc-502; Santa Cruz Biotechnology, USA), and anti-IL-6 (1:500, ab9324; Abcam). Subsequently, the sections were incubated with the proper secondary antibody [1:200, polyclonal rabbit anti-mouse biotinylated (E0354; Dako, USA), or 1:200, polyclonal swine anti-rabbit biotinylated (E0353; Dako)], for 30 min at room temperature. After that, sections were incubated for 30 min with an avidin-biotin complex (Vector Laboratories, UK) and then revealed with the DAB substrate (3,30-diaminobenzidine tetrahydrochloride; Dako, USA). All the sections were counterstained with Harris haematoxylin.

Tissue slides immunohistochemically stained for Ki-67, performed as part of routine practice to determine the tumor grade, were retrieved from the pathology department archives and used for morphological analysis.

3.4.4.3. Immunohistochemical data analysis

Haematoxylin and eosin (H&E) stained slides were used for tumor area delimitation based on morphological criteria by experienced pathologists with no access to patients' clinical information. This area delimitation was then

transferred to the immunohistochemistry stained slides. After immunohistochemistry, slides were scanned using the image acquisition Olympus VS110 virtual slide scanning system (Olympus, Japan) and captured with a magnification of 20× using the image acquisition software VS-ASW (Olympus). Images were analysed using the image processing software FIJI (version for Windows; National Institutes of Health, USA). The tumor area was selected using FIJI freehand tool, for the study of the expression of Ki-67, FOXM1 and IGF1R. A peritumoral area of 5 mm distant from the tumor and from 5 mm until the end of the tissue was delimited using the ROI Manager Tool of FIJI to evaluate IL-6 expression. Using FIJI colour deconvolution plugin (H Dab), the separation of the stained area from the initial image, based in the RGB (red, green and blue) system was performed. Then, the stained area with the IGF1R, FOXM1 and Ki-67 antibodies in the total tissue area of the tumor and the stained area with the IL-6 antibody in the adjacent tissue, were quantified as previously described (Pereira, Pereira et al. 2017).

3.4.4.4. Statistical analysis

Qualitative variables are expressed as number of cases and percentage (%), and the quantitative variables are expressed as mean ± standard error of the mean. 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 normality. To correlate the different groups, a Pearson or a Spearman correlation was used depending on the sample's normality. A p value <0.05 was considered statistically significant. All statistical analyses were performed with the Graph-Pad Prism software version 7.00 (GraphPad, USA) and IBM SPSS Statistics version 24 (IBM, USA), both for Windows.

3.4.5 Results

3.4.5.1. Expression of Ki-67, FOXM1, IGF1R and IL-6 markers in patients with and without MetS.

All tumors expressed Ki-67, FOXM1 and IGF1R, although the percentage of stained area was highly variable (Fig. 13). FOXM1 and IGF1R positive cells were found to be evenly distributed throughout the tumor tissue.

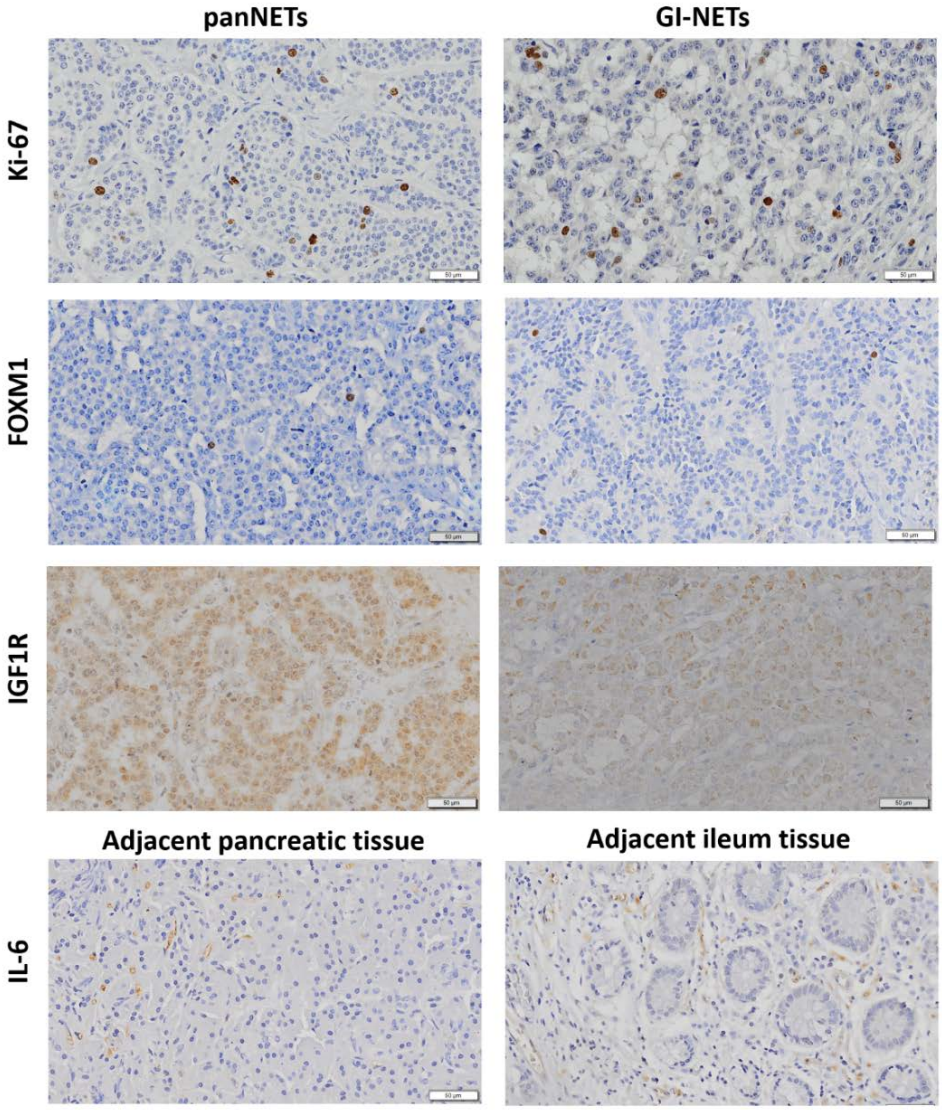

Figure 13. Immunohistochemistry staining for Ki-67, FOXM1 and IGF1R and IL-6 in panNEN and GI-NEN, and for IL-6 in the pancreatic and ileum tissue adjacent to the panNEN or GI-NEN, respectively.

The percentages of the stained tumor areas, both panNEN and GI-NEN, for all the molecular markers Ki-67, FOXM1 and IGF1R were not significantly different when patients with or without MetS were compared (panNEN: p=0.99 for Ki-67, p=0.61 for FOXM1, and p=0.65 for IGF1R; GI-NEN: p=0.62 for Ki-67, p=0.96 for FOXM1, and p=0.19 for IGF1R) (Fig. 14).

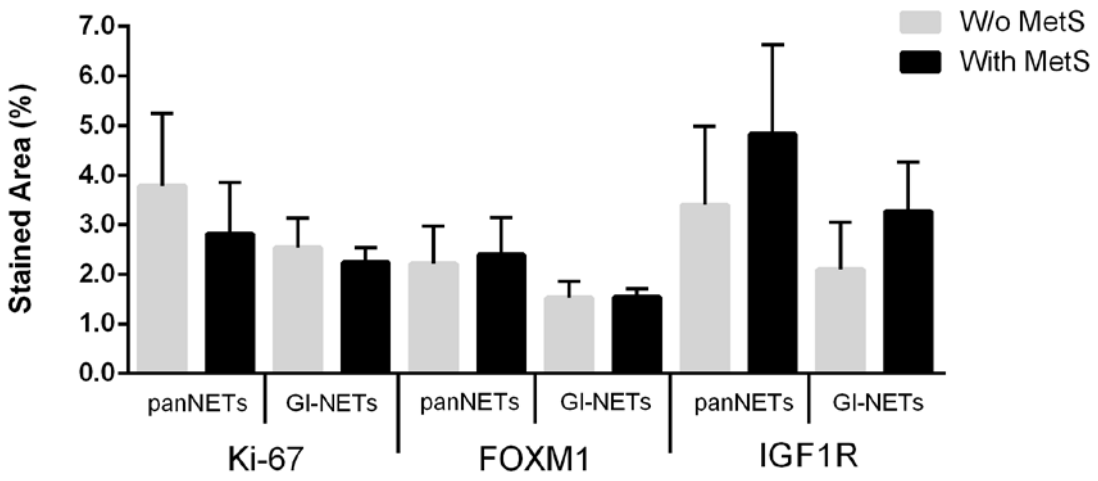

Figure 14. Percentage of the peritumoural areas (≤5 and >5 mm) stained for Ki-67, FOXM1 and IGF1R in pancreatic (panNEN) and gastrointestinal neuroendocrine neoplasia (GI-NEN) of patients with or without (W/o) metabolic syndrome (MetS).

Nevertheless, the percentage of IGF1R stained area was higher in panNEN and GI-NEN of patients with MetS (Fig. 14).

The percentage of IL-6 stained in the peritumoral area was assessed within 5 mm from the tumor limit and from this limit until the tissue edge. IL-6 was found to be expressed in the peritumoral pancreatic and intestinal stroma, mainly in endothelial, fibroblasts and immune cells. The results showed that the percentage of IL-6 stained area in peritumoral areas of WD GEP-NEN was not significantly different when patients with or without MetS were compared. No difference in peritumoral IL-6 stained area was observed in panNEN with or without MetS [IL-6 (≤ 5 mm): 0.024 ± 0.009 (with MetS) vs 0.017 ± 0.011 (without

MetS), p=0.56;IL-6 (>5 mm): 0.015 ± 0.007 (with MetS) vs 0.015 ± 0.004 (without MetS), p=0.61]; nor in GI-NEN [IL-6 (≤ 5 mm): 0.027 ± 0.005 (with MetS) vs 0.021 ± 0.006 (without MetS), p=0.40; IL-6 (>5 mm): 0.019 ± 0.002 (with MetS) vs 0.025 ± 0.006 (without MetS), p=0.85] (Fig. 15).

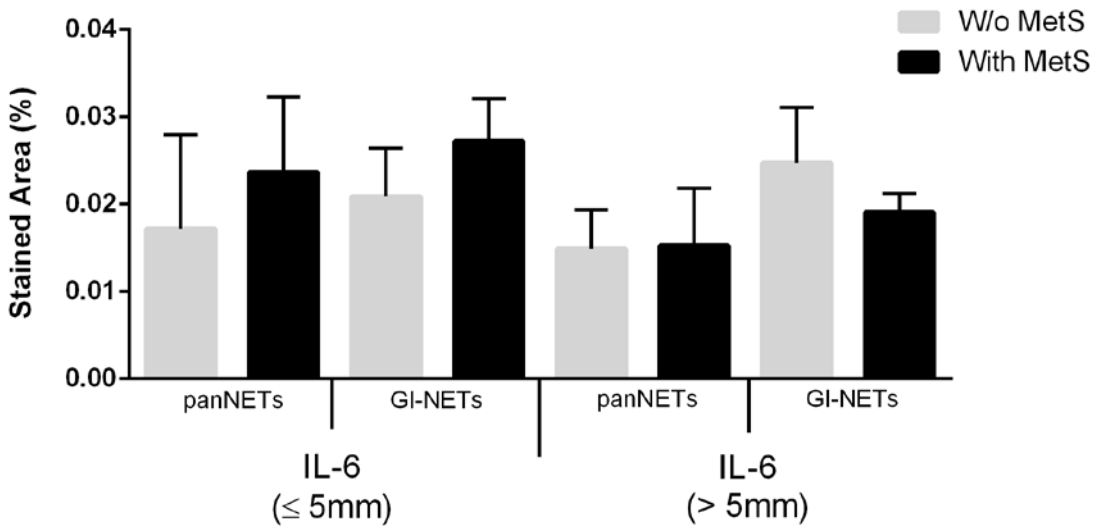

Figure 15. Percentage of the peritumoural areas (≤ 5 and >5 mm) stained for IL-6 in pancreatic (panNEN) and gastrointestinal neuroendocrine neoplasia (GI-NEN) of patients with or without (W/o) metabolic syndrome (MetS).

3.4.5.2 - Expression of Ki-67, FOXM1, IGF1R and IL-6 markers in patients with or without MetS components

The percentages of the stained tumor areas for Ki-67, FOXM1 and IGF1R markers, were not significantly different between patients with or without each individual MetS component, both for panNEN and GI-NEN (Table XX).

Table XX. Percentage of the tumor area stained in the immunohistochemical markers, Ki-67, FOXM1 and IGF1R, in pancreatic and gastrointestinal neuroendocrine neoplasia.

	MetS Components	Ki-67	FOXM1	IGF1R
Pancreatic Neuroendocrine Neoplasia	**BP normal:raised[1]**	2.74±1.28:3.73±1.27 p = 0.61	2.06±0.77:2.68±0.85 p = 0.62	3.18±1.67:5.51±1.98 p = 0.47
	Fasting plasma glucose normal:raised[2]	3.78±1.46:3.04±1.20 p = 0.91	2.67±0.67:2.28±0.89 p = 0.47	6.18±4.34:4.01±0.87 p = 0.51
	Triglycerides normal:raised[3]	4.06±1.30:2.85±1.24 p = 0.48	2.35±0.69:2.49±0.89 p = 0.76	2.88±1.10:6.22±2.30 p = 0.27
	HDL normal:low[4]	3.37±1.20:3.29±1.43 p = 0.84	2.02±0.61:2.85±1.01 p = 0.50	4.08±1.31:5.26±2.47 p = 0.71
	Central obesity absent:present[5]	3.02±1.76:2.55±0.73 p = 0.90	2.21±1.06:1.83±0.26 p = 0.64	3.90±2.60:4.98±2.08 p = 0.80
Gastrointestinal Neuroendocrine Neoplasia	**BP normal:raised[1]**	2.42±0.57:2.30±0.30 p = 0.84	1.71±0.37:1.46±0.15 p = 0.77	2.83±1.15:2.94±0.96 p = 0.51
	Fasting plasma glucose normal:raised[2]	2.21±0.47:2.39±0.33 p = 0.99	1.38±0.32:1.61±0.17 p = 0.49	3.25±1.13:2.75±0.96 p = 0.77
	Triglycerides normal:raised[3]	2.48±0.48:2.16±0.38 p = 0.56	1.70±0.22:1.34±0.21 p = 0.23	3.70±1.25:1.93:0.56 p = 0.11
	HDL-c normal:low[4]	1.89±0.34:2.70±0.39 p = 0.13	1.27±0.18:1.76±0.22 p = 0.11	2.12±0.68:3.55±1.23 p = 0.28
	Central obesity absent:present[5]	2.39±0.42:2.30±0.36 p = 0.87	1.51±0.26:1.56±0.19 p = 0.86	2.12±0.68:3.55±1.23 p = 0.28

[1]Systolic BP ≥ 130 mm Hg or Diastolic BP ≥ 85 mm Hg; [2] Fasting plasma glucose ≥ 100 mg/d; [3]Triglycerides ≥ 150 mg/dL; [4]HDL-c< 40 mg/dL (male) or < 50 mg/dL (female); [5]Waist circumference ≥ 102cm (male) or ≥ 88cm (female); MetS- Metabolic Syndrome; BMI – Body mass index; BP- Blood Pressure; HDL-c- High-density lipoprotein.

The percentage of the peritumoral area stained for the IL-6 marker assessed at two different distances from the tumor was not significantly different between patients with or without several of the MetS individual components, namely high FPG, high BP or raised TG, for both panNEN and GI-NEN (Table XXI).

Table XXI. Percentage of the peritumoral area immunohistochemically stained with IL-6 at two different distances from pancreatic and gastrointestinal neuroendocrine neoplasia margins.

	MetS Components	IL-6 (≤5mm)	IL-6 (>5mm)
Pancreatic Neuroendocrine neoplasia	**BP normal:raised[1]**	0.019±0.010:0.022±0.0091 p = 0.81	0.015±0.0036:0.014±0.0067 p = 0.35
	Fasting plasma glucose normal:raised[2]	0.019±0.010:0.022±0.0090 p= 0.91	0.014±0.0044:0.015±0.0065 p = 0.89
	Triglycerides normal:raised[3]	0.019±0.012:0.022±0.0081 p = 0.84	0.010±0.0047:0.018±0.0060 p = 0.35
	HDL normal:low[4]	0.024±0.011:0.017±0.0072 p = 0.84	0.013±0.0038:0.016±0.0077 p =0.69
	Central obesity absent:present[5]	0.022±0.013:0.016±0.0079 p = 0.71	**0.019±0.0025:0.0078±0.0019 p = 0.01**
Gastrointestinal Neuroendocrine neoplasia	**BP normal:raised[1]**	0.022±0.0055:0.026±0.0049 p = 0.68	0.025±0.0062:0.018±0.0022 p = 0.60
	Fasting plasma glucose normal:raised[2]	0.025±0.0053:0.024±0.0050 p = 0.47	0.023±0.0058:0.019±0.0022 p = 0.77
	Triglycerides normal:raised[3]	0.024±0.0050:0.026±0.0058 p = 0.75	0.020±0.0026:0.021±0.0044 p = 0.70
	HDL normal:low[4]	**0.018±0.0050:0.030±0.0050 p = 0.02**	0.017±0.0027:0.024±0.0040 p = 0.17
	Central obesity absent:present[5]	0.019±0.0047:0.029±0.0052 p = 0.13	0.022±0.0047:0.020±0.0028 p = 0.96

[1]Systolic BP ≥ 130 mm Hg or Diastolic BP ≥ 85 mm Hg; [2] Fasting plasma glucose ≥ 100 mg/d; [3]Triglycerides ≥ 150 mg/dL; [4]HDL< 40 mg/dL (male) or < 50 mg/dL (female); [5]Waist circumference ≥ 102cm (male) or ≥ 88cm (female); MetS- Metabolic Syndrome; BMI – Body mass index; BP- Blood Pressure; HDL- High-density lipoprotein.

However, in GI-NEN the percentage peritumoral area at 5 mm distance or less from the tumor limit stained for IL-6 was significantly higher in the subset of patients with low HDL-c when compared to patients with normal HDL-c (0.030 ± 0.0050 vs 0.018 ± 0.0050; $p<0.05$) (Table XXI). In addition, in panNEN the

percentage of peritumoral area stained for IL-6 at 5 mm from the tumor limit until the end of the tissue was significantly higher in patients without central obesity when compared with patients with central obesity (0.019 ± 0.0025 vs 0.0078 ± 0.0019; $p<0.05$) (Table XXI).

3.4.5.3 Ki-67, FOXM1, IGF1R and IL-6 expression and tumor characteristics

The percentages of the stained tumor areas for Ki-67, FOXM1 and IGF1R markers did not differ according to the different tumor's characteristics (Table XXII).

Table XXII. Percentage of the tumor area stained in the immunohistochemical markers, Ki-67, FOXM1 and IGF1R, in pancreatic and gastrointestinal neuroendocrine neoplasia, according to tumors characteristics

	Tumors characteristics	Ki-67	FOXM1	IGF1R
Pancreatic Neuroendocrine neoplasia	**WHO grade G1:G2**	2.54±0.83:4.53±1.83 p=0.48	2.15±0.51:2.86±1.31 p=0.99	5.42±2.50:3.88±1.12 p=0.99
	Functionality non-functioning:functioning	3.04±0.86:5.45±3.51 p=0.33	1.90±0.43:4.05±2.69 p=0.50	3.53±0.91:3.32±1.65 p=0.64
	Tumor progression without:with	3.97±1.52:2.70±0.99 p=0.67	2.53±1.07:2.33±0.59 p=0.67	4.33±0.98:5.25±3.22 p=0.69
Gastrointestinal Neuroendocrine neoplasia	**WHO grade G1:G2**	2.21±0.28:2.93±0.81 p=0.38	1.53±0.17:1.61±0.39 p=0.75	2.85±0.84:3.18±165 p=0.76
	Functionality non-functioning:functioning	2.55±0.67:2.27±0.29 p=0.89	1.97±0.33:1.40±0.16 p=0.19	6.22±2.62:1.86±0.35 p=0.35
	Tumor progression without:with	2.14±0.39:2.5±0.38 p=0.51	1.35±0.15:1.69±0.24 p=0.27	1.68±0.30:3.91±1.29 p=0.31

The percentage of peritumoral area stained for IL-6 was significantly higher at a distance of 5 mm from the tumor until the end of the tissue in GI-NEN that progressed at least once after the initial treatment (Table XXII, Fig. 16).

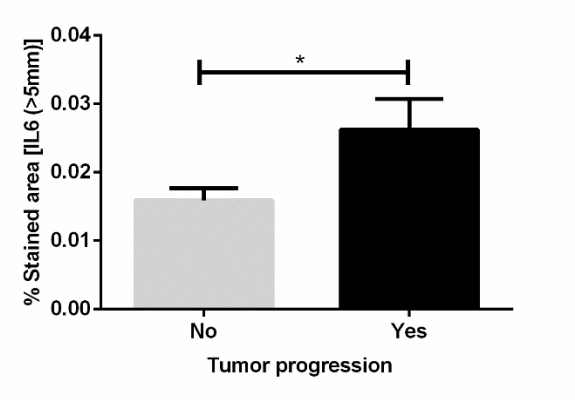

Figure 16. *Percentage of peritumoural area stained for IL-6 at a distance of 5 mm from the tumour until the end of the tissue in GI-NEN, according with tumour progression (Mann-Whitney U test: *p=0.05).*

No other correlation was observed between characteristics of GEP-NEN and peritumoral IL-6 staining (Table XXIII).

Table XXIII. Percentage of the peritumoral area immunohistochemically stained with IL-6 at two different tumor distances according to tumors characteristics.

	Tumors characteristics	IL-6 (≤5mm)	IL-6 (>5mm)
Pancreatic Neuroendocrine neoplasia	**WHO grade G1:G2**	0.024±0.009:0.016±0.010 p=0.33	0.013±0.003:0.018±0.010 p=0.83
	Functionality non-functioning:functioning	0.020±0.008:0.028±0.018 p=0.67	0.012±0.003:0.026±0.022 p=0.89
	Tumor progression without:with	0.024±0.011:0.018±0.008 p=0.94	0.016±0.008:0.015±0.003 p=0.67
Gastrointestinal Neuroendocrine Neoplasia	**WHO grade G1:G2**	0.021±0.027:0.021±0.007 p=0.68	0.026±0.004:0.020±0.004 p=0.94
	Functionality non-functioning:functioning	0.018±0.004:0.022±0.003 p=0.45	0.027±0.010:0.024±0.004 p=0.88
	Tumor progression without:with	0.026±0.005:0.024±0.005 p=0.52	**0.026±0.004:0.016±0.002 p=0.03**

3.4.5.4. Molecular correlations

Ki-67 and FOXM1 were found to be positively correlated both in panNEN (R=0.648; p<0.05) (Fig. 17A) and in GI-NEN (R=0.606; p<0.001) (Fig. 17B). In addition, a statistically significantly positive correlation between IGF1R and FOXM1 in GI-NEN was found (R=0.608; p<0.001) (Fig. 17C).

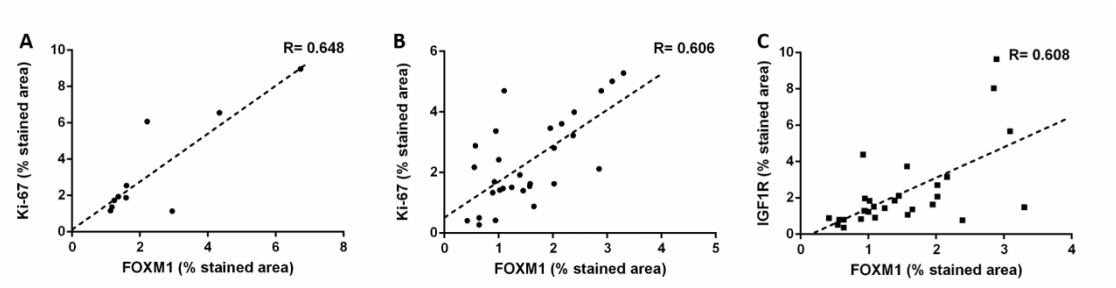

Figure 17. *(A) Correlations between FOXM1 and Ki-67 in pancreatic neuroendocrine neoplasia. (B) Correlations between FOXM1 and Ki-67 and (C) FOXM1 and IGF1R in gastrointestinal neuroendocrine neoplasia.*

No other significant correlations were found between the expression pattern of the studied proteins in panNEN or GI-NEN.

3.4.6 Discussion

MetS is a well-established RF for different types of cancers (Braun, Bitton-Worms et al. 2011, Arcidiacono, Iiritano et al. 2012, Esposito, Chiodini et al. 2012). More recently, the potential link between MetS and WD GEP-NEN has also been highlighted, since MetS and several individual components of MetS, namely FPG, WC, and dyslipidaemia were found to be more frequent in patients with WD GEP-NEN then in the general population (Santos, Santos et al. 2018). However, no molecular links for this pathological association that could provide novel information on the mechanisms of disease and lead to the development of targeted interventions were identified. Thus, the main aim of the research herein was to gain further insight into putative molecular links that could provide

pathological rationale for the association of WD GEP-NEN and MetS or any individual component of MetS. To achieve this goal, FOXM1 and IGF1R were selected as markers of the molecular pathways involved in GEP-NEN`s biology and IL-6 was chosen as to evaluate the inflammatory environment in the periphery of the tumor. Despite the rationale for the potential involvement of these molecular pathways in the likelihood of MetS being associated with WD GEP-NEN, our results were not able to demonstrate any significant association between IGF1R and FOXM1 expression in WD GEP-NEN and MetS or any of the MetS individual components, which could suggest that expression of these molecules is not influenced by the presence of MetS. Nevertheless, it should be noted that IGF1R expression in WD GEP-NEN, both in panNEN and GI-NEN, although not reaching statistical significance was numerically higher in tumors of patients with MetS when compared to patients without MetS, suggesting that higher insulin levels in patients with MetS are likely to be involved in the upregulation of IGF1R and subsequent pathway activation. Ki-67 is a well-known cell proliferation marker routinely used in clinical practice for pathological staging of several tumors including GEP-NEN. In our study, MetS or any of the MetS individual components were not found to be associated with a higher Ki-67 expression in WD GEP-NEN to suggest that MetS could influence cell proliferation rate and eventually influence the tumor biological and clinical behaviour. Nonetheless, a positive correlation between Ki-67 and FOXM1 expression in WD GEP-NEN, both panNEN and GI-NEN, was similar to a previous report involving GEP-NENs (Briest, Berg et al. 2015). In GI-NENs, FOXM1 and IGF1R expression were found to be positively correlated. This correlation further supports that FOXM1 expression is stimulated and activated by IGF1R in GI-NEN. Chronic inflammatory conditions are well recognised risks for cancer. Inflammatory bowel disease (IBD) is associated with increased risk of GEP-NEN (Sigel and Goldblum 1998, Macarthur, Hold et al. 2004, Cigrovski Berkovic, Cacev et al. 2014, Barral, Dohan et al. 2016), further suggesting that chronic inflammation within the gastrointestinal tract could promote hyperplasia and neoplastic transformation of neuroendocrine cells (Cigrovski Berkovic, Cacev et al. 2014). In addition to the widely accepted role of inflammation in tumorigenesis, it has become evident that the inflammatory microenvironment is a key component in tumor biology (Wang, Zhao et al. 2017). Since IL-6 is a

cytokine often expressed in tumor surrounding tissues, and its systemic levels have been found to be increased in patients with obesity and MetS (Monteiro and Azevedo 2010, Fisher, Appenheimer et al. 2014, Tanaka, Narazaki et al. 2014), IL-6 expression surrounding the tumor was elected as a mean to assess inflammatory activity in WD GEP-NEN of patients with potentially different systemic inflammatory profiles. The area that surrounds a tumor with the extensively described anti-inflammatory properties of HDL-c (Barter, Nicholls et al. 2004, Navab, Yu et al. 2007). Besides that, a higher IL-6 expression in the peritumoral area of GI-NEN was observed in tumors of patients with progressive disease. So, our data further support ability to influence tumor environment has not yet been strictly defined and is potentially variable depending on the type and location of cancer. Thus, it is not surprising that previous studies have used a wide range of distances from the tumor limit, spanning from few millimeters to 1 cm wide, that were defined as adjacent tumor tissue (Mangiola, Lama et al. 2007, Zhuang, Shen et al. 2013, Balsat, Signolle et al. 2014, Pak, Jo et al. 2015). In our study, whenever available a maximum distance of 5 mm from the tumor limit was selected as the definition of peritumoral tissue to assess IL-6 expression in the tumor microenvironment (Zhuang, Shen et al. 2013, Balsat, Signolle et al. 2014). IL-6 expression in WD GEP NEN`s peritumoral tissue of patients with and without MetS was not found to be significantly different, both within the 5 mm perimeter from the tumor limit or 5 mm or higher until the tissue limit. Since MetS is a known chronic low-grade inflammatory state (Monteiro and Azevedo 2010), IL-6 expression surrounding WD GEP NEN of patients with MetS was expected to be higher, which was not corroborated by our findings. Nonetheless, it should be noted that IL-6 expression in WD GEP-NEN, both in panNEN and GI-NEN, despite not reaching statistical significance was numerically higher in the area within 5 mm of the limit of tumors of patients with MetS when compared to patients without MetS. In addition, in patients with panNEN and central obesity, a lower peritumoral IL-6 expression was noticed in contrast to what was previously described in the literature for tumors other than GEP-NEN (Monteiro and Azevedo 2010). However, IL-6 expression in the peritumoral area of GI-NEN was significantly higher in patients with low HDL-c when compared to tumors in patients with normal HDL-c, suggesting that HDL-c could be an important modulator of the inflammatory environment in GI-NEN, unsurprisingly given the

role of chronic inflammation in the modulation of GI-NEN`s behaviour, as previously proposed (Cigrovski Berkovic, Cacev et al. 2014, Walenkamp, Crespo et al. 2014). Despite the novel findings brought by conducting this study, a few limitations that could impact data interpretation deserve to be considered. Although WD GEP-NEN are the second most frequent digestive neoplasia, these tumors are still not very frequent, thus the small number of tumors available for analysis in our series is understandable, in particular given that this originated from a single centre. Indeed, the small numbers in our series represent a limitation to the extent of the conclusions retrieved, as compared to what could be expected if a larger sample multicentre series was available. In the presence of a larger sample size some of the trends observed in this study could eventually reach significance.

Nevertheless, novel pathways of research were unravelled, in particular leading to the need to focus on the detailed characterisation of the role of local and systemic inflammatory status on GI-NEN`s biology.

3.4.7 Conclusion

The influence of MetS in the molecular inflammatory and metabolic profile of WD GEP-NEN was assessed. IL-6 expression in tissues surrounding GI-NEN was influenced by MetS features and positively associated with disease progression. In contrast, FOXM1 and IGF1R expression in WD GEP-NEN were not influenced by MetS.

In summary, our findings suggest that the inflammatory status could be a potential mechanism linking MetS and GI-NEN in addition to having a putative role in the modulation of GI-NEN behaviour.

Chapter 4 - FINAL DISCUSSION

"… Concepts are by nature relational and all knowledge is relational knowledge… /… the similarity that constitutes the analogy is not between the phenomena themselves but between the relations of these phenomena"

In "On Understanding Maxwell on the methods of illustration and Scientific Metaphor" Jordi Cat, 2001, citando James Clerk Maxwell in Studies in History and Philosophy of Modern Physics, 1856

4.1 Mechanisms of the Endocrine Regulation – Feed-back Mechanisms in Endocrinology, Inflammation and Cancer

Before the 6th century BC, nature was regarded as the work of God, nature was static.Then, Heraclitus and other philosophers laid the foundation of modern science as they introduced change as a new way of thinking nature. According to Heraclitus, "everything flows like the continuous of the flickering flame, there was nothing permanent in a fire" (Zajicek 1993). The ultimate task of all organ systems of the body is maintaining homeostasis through sophisticated integrating systems that regulate the function of individual tissues and organs developed during evolution. If nervous system is responsible for acute reactions, slower and long-term adaptation in response to internal and external environment changes are an attribute of the neuroendocrine system. Immune system is also involved in situations where homeostasis is compromised (Verburg-van Kemenade, Cohen et al. 2016).

So, endocrinology is a dynamic and aggregating speciality, involving most of the "actors" responsible for the permanent adaptation of the living cells and organs to the changes of macro and microenvironment they are continuously facing.

The essence of this neuroendocrine adaptation are the endocrine feed-back mechanisms, which could be either negative or positive. In the first case, feedback control permits to maintain homeostasis because there is a self-regulation of the changes induced by a hormone in the target tissue that can cause reverse inhibition of that hormone. In order to prevent hypersecretion of thyroid hormones, there is a negative feedback on the hypothalamus and the hypophysis that inhibits the secretion of TRH and TSH. If glucose lowers in blood, there is a signal for the pancreas to secrete less insulin in order to maintain normoglycemia. On the contrary, when target tissues detect low hormone levels, a compensatory positive feedback is triggered in order to restore the normal homeostasis by increasing stimulating hormones. When low levels of cortisol are detected, positive feedback acts both in the hypothalamus and the hypophysis, increasing ACTH production in order to stimulate adrenal secretion of cortisol. For instance, Nelson`s syndrome is often found in patients submitted to bilateral adrenalectomy due to a pituitary tumor caused by adrenocorticotrophic stimulation secondary to the absence of adrenal produced cortisol (Banasiak and Malek 2007). Chronic lymphocytic thyroiditis (CLT), so called Hashimoto thyroiditis, is an autoimmune disease in which lymphocytic infiltration of thyroid cells leads to an atrophy of the glandule with subsequent hypofunction and hypothyroidism. Further compensatory feedback endocrine axis mechanisms develop, to try to compensate the low thyroid hormone secretion, leading to an excessive secretion of TSH by the pituitary. Micropapillary thyroid cancers are frequently found in Hashimotos`s disease and some authors believe on the association of CLT with an higher risk for papillary thyroid cancer, particular in those individuals with high TSH levels (Fugazzola, Colombo et al. 2011, Lee, Kim et al. 2013).

In the same way, when blood glucose levels increase, a positive feedback on the pancreas stimulates insulin secretion to avoid hyperglycaemia. In healthy individuals these physiological mechanisms are intact and glucose homeostasis is maintained. The problem arises when there is a chronic hormone deficiency despite continuous stimulation of target cells, through feedback mechanisms with the objective of restoring normal hormone levels. At that time, compensation cannot overcome the inbalance and disease emerges.

Another example of the importance of the endocrine feed-back mechanisms in hoemostasis and cancer are Ratcliffe`s studies. His work on the regulation of haematopoietic growth factor erythropoietin as a model for adaptative mechanisms to hypoxia and its implications in the physiology of cancer were awarded with 2019`s Medicine Nobel Prize (Ratcliffe 2013).

So, before disease is declared, there is a long-term state of subclinical compensatory phase where all the mechanisms are working for the homeostasis, e.g. the WOB is playing! As Claude Bernard stated (Canguilhem 2008), the transition of normality to pathology proceeds in a continuous and reversible fashion. At the point when for any reason compensation is not enough, disease becomes manifest. When hyperinsulinism cannot compensate IR anymore, because pancreatic beta cells are exhausted, diabetes supervenes.

The knowledge of the mechanisms responsable for the development of T1-GEN (Chapter 1.6.4) made me wonder if a similar mechanism could be responsible for neuroendocrine neoplasia in other locations.

Indeed, there are several findings that suggest common mechanisms between gut peptide hormones and cancer, namely in NEN, where hormone feed-back regulation seems to have an important role.

Besides its role in T1-GEN development, gastrin also promotes gastric adenocarcinoma formation but only in conjunction with other co-factors, such as a mutant cell phenotype (Watson, Grabowska et al. 2006) or bacterial pathogenicity factors, or through synergizing with inflammatory events associated with H. pylori infection (Wang, Dangler et al. 2000).

CCK has been demonstrated to be a potent stimulator for pancreatic growth (Thomas, Hellmich et al. 2003) with a role on pancreatic carcinogenesis that has been demonstrated in experimental (Smith, Kramer et al. 1991) and animal studies (Heald, Kramer et al. 1992).

GLP-2 is a proglucagon-derived peptide with a trophic effect on intestinal mucosae. It stimulates the growth of small intestine tissue for preventing intestinal

atrophy associated with prolonged total parenteral nutrition (Martin, Wallace et al. 2004) or chemotherapy associated mucositis (Kissow, Viby et al. 2012).

PYY, a peptide secreted in the small intestine, well known for its anorexigen effect by action on the mechanisms of satiety, has also a trophic effect on small intestine and colonic mucosa of both rat and mouse (Gomez, Zhang et al. 1995).

On the other side, inhibitory somatostatin action with anti-proliferative effects on gastrointestinal and pancreatic neuroendocrine neoplastic cells led to the approval of SA for the treatment of functioning and non-functioning GEP-NEN (Rinke, Muller et al. 2009, Caplin, Pavel et al. 2014). These agents also demonstrated to be effective in non-neuroendocrine neoplasia such as breast cancer (Pollak 1997) and other digestive malignancies like pancreatic adenocarcinoma as well as neuroblastoma in children (Keskin and Yalcin 2013).

The presence of serotonin excess in SI-NEN is well known. NEN arising from jejunum and ileum may secrete excessive levels of 5-HT, which originates a carcinoid syndrome characterized by secretory diarrhoea, flushing, abdominal pain and right heart valvular dysfunction (Kaltsas, Besser et al. 2004). Midgut NEN are usually diagnosed later in the course of the disease, when 40-60% are already metastized (Strosberg, Halfdanarson et al. 2017). However, the natural history of NEN reveals that symptoms are usually present for more than 12 years before diagnosis with vague abdominal symptoms often interpreted as irritable bowel syndrome (IBS) (Vinik, Silva et al. 2009).

Interestingly, the role of 5-HT in the pathogenesis of IBS has been well established. Enteric 5-HT is responsible for the secretion, motility and perception of the bowel. Higher 5-HT availability is commonly associated with depressed serotonin transporters (SERT) mRNA in patients with IBS compared with healthy controls. SERT expression is in turn modulated by several factors that include SERT gene polymorphisms, microRNAs, immunity and inflammation, gut microbiota, growth factors, among others (Jin, Cao et al. 2016). The influence of previous infection, inflammation and altered microbiota in IBS is well documented (Enck 2019) in nearly 10% of the cases.

The association of IBS with post bacterial, viral or parasitic infeccion is also well established and related to visceral hypersensivity and GI dysmotility, even with no endoscopic or microscopic lesions. An hypothesis for that has been recently postulated (Beatty, Bhargava et al. 2014). Pathogens disrupt the mucosal barrier and subsequently mucosal immune cells are persistently activated in result of increased exposure to luminal antigens. This low-grade mucosal inflammation is able to affect immune system, endocrine cell behaviour, and subsequently visceral sensivity and GI motility, thus leading to post-infecctious IBS. Curiously, midgut carcinoid patients display tumor tissue infiltration CD4+ and CD8+ T cells in the presence of regulatory CD4+FoxP3+ cells (Vikman, Sommaggio et al. 2009).

In autoimmune intestinal diseases like celiac disease (CD), the villous atrophy and cryptic hyperplasia is associated with an increase in the number of cells containing 5-HT and patients with untreated CD have a significant increase in postprandial platelet-poor plasma 5-HT levels compared with controls, which correlates with postprandial dyspepsia (Coleman, Foley et al. 2006). These authors suggest that serotonin excess may mediate dyspeptic symptoms in untreated CD.

Although the relationship between NEN and CD has not been formally addressed in experimental studies, there are some literature reports of patients with concomittant CD and ileal NEN (Sottile, Percopo et al. 2001, Kimchi, Broide et al. 2005). Also, a study about primary small-bowell malignancy association with CD in UK between 1998-2000 (n=395) found 20% of carcinoid tumors (Howdle and Holmes 2004).

Although the importance of incretins as gastrointestinal mediators of intestinal endocrine functions is well recognized, their role in the pathogenesis of NENs is yet to be established. However, GIP receptors were identified in most GEP-NEN and bronquial NEN (Waser, Rehmann et al. 2012). GLP1-receptors were also found in 90% of insulinomas and ^{68}Ga-labelled exendin-3 has been developed as a new agent for ^{68}Ga-DOTA-exendin-3 PET for detection of insulinomas, which generally lack somatostatin receptor subtype 2 that are not detected with

conventional somatostatin analogues scintigraphy (Waser, Rehmann et al. 2012).

Connection between incretins and endocrine hyperplasia is suggested by secondary pancreatic β-cell hyperplasia due to bariatric Roux-en-Y surgery in morbid obesity, called non-insulinoma pancreatogenous hypoglycaemia (NIPH) (Anderson, Nostedt et al. 2016) and describes hypoglycemia syndromes in adults without evidence of insulinoma (Guimaraes, Rodrigues et al. 2015). Interestingly, in patients submitted to gastric bypass surgery, post-prandial incretin levels raise three-to-five-fold (Laferrere 2009). Case reports describing neuroendocrine neoplasia producing GLP1 and somatostatin and GLP1, GLP2 and PYY have been published (Todd, Stanley et al. 2003) (Byrne, McGregor et al. 2001) and a neuroendocrine tumor producing GLP-1 and GLP-2 in a patient with intractable constipation and intermittent vomiting was also described (Brubaker, Drucker et al. 2002).

Besides, TAM and high peritumoral CD4+ cell were associated with a worse disease free survival in panNEN, thus reflecting immune–inflammatory reactions also in this location (Cai, Michelakos et al. 2019).

In summary, interactions between inflammation of both intestinal and pancreas tissues and neuroendocrine cell alterations mediated by peptides and hormones that could lead to transformation into neuroendocrine neoplasia, is an unexplored world that opens an interesting field for investigation.

Overall, given the above mentioned lines of evidence, it is reasonable to believe that in a similar manner as observed in other neoplasia, the pathogenic mechanism responsible for sporadic gut and pancreatic neuroendocrine neoplasia also involves chronic inflammation caused by infection, autoimmunity or other unidentified mechanisms that could lead to the depletion of some substance, peptide or hormone, with feed-back stimulation of another one in order to reach a new homeostasis.

In the case of SI-NEN, a cascade ressembling the one observed for T1-GEN is postulated. Chronic inflammation (inflammatory bowell disease? Post-infeccious colitis?, IR?) would lead to intestinal atrophy, increased mucosal permeability and altered microbiota, which in turn would decrease some kind of peptide or hormone that through a positive feed-back mechanism, would increase serotonin secretion leading to consequent chronic stimulation of intestinal neuroendocrine cells. Whenever this proliferative stimulus is able to remain uncompensated or gains autonomy, a midgut NEN would then supervene (Fig. 18).

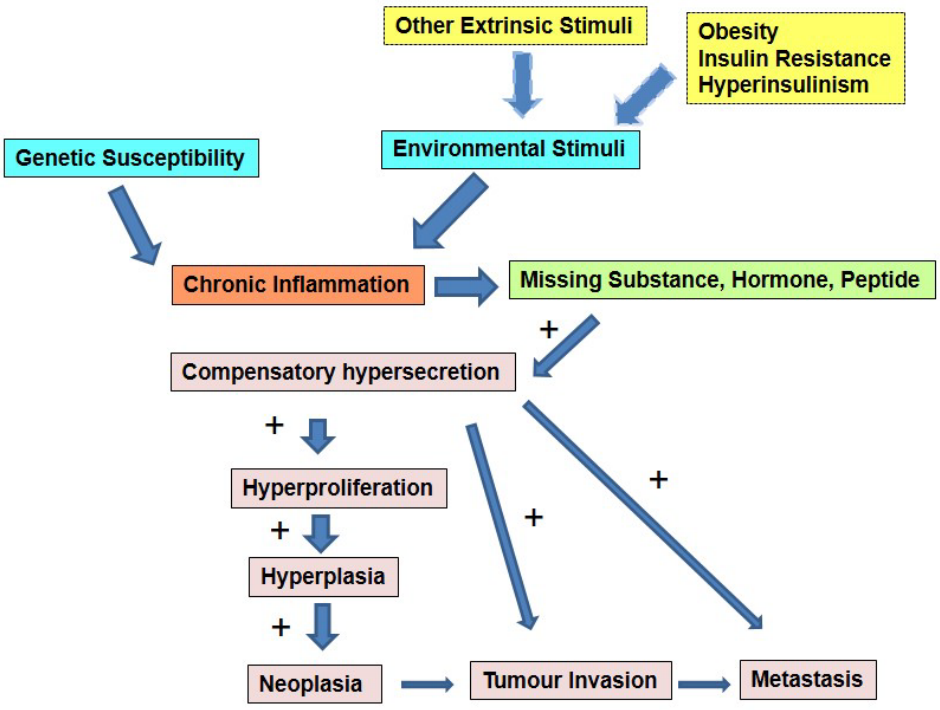

Figure 18. Proposed model for endocrine feed-back mechanisms linking inflammation, hormones and cancer pathogenesis

The role of obesity and diabetes in this process is unknown, but as other chronic subclinical inflammatory conditions, they share inumerous pathophysiological mechanisms with carcinogenesis, namely in neuroendocrine neoplasia. Probably cancer, and in this case NEN, arise from a multicity of RFs that act together since the pre-clinical phase of the disease. Independently of whether investigation is

basic or clinical, the main principle of the human body functioning is homeostasis, the way that body reacts to changes in environmental stimuli, trying to reach a new equilibrium (Cannon 1939).

In the first phase there is an adaptative compensation trying to maintain homeostasis. Later on the course of the disease, WOB fails and illness becomes manifest.The more precise is the knowledge on cancer aethiology and the interaction between intrinsic and multiple exogenous RFs that lead to neoplastic transformation of cells (Wu, Zhu et al. 2018), more able we are to intervene towards cancer prevention and treatment.

4.2 Neuroendocrine Tumors and Chronic Inflammation: A Model for the Association Between Endocrine Feedback Mechanisms and Cancer Pathogenesis

The theme of the present dissertation is about neuroendocrine neoplasia. Yet it was not solely focused on this rare malignancy. The objective of my research was to try to integrate a rare disease in a more global context, which is the relationship between three of the most important NCD epidemics - obesity, diabetes and cancer. At the same time, I intended to find some clues, in order to direct research towards understanding the pathogenesis of neuroendocrine tumors in a more comprehensive manner.

I think one of the main messages of the present dissertation is that we have to change the paradigms on our way of looking at cancer, which of course has to be assumed as a chronic disease, but in a global and multidisciplinary way, with interaction between specialities as a part of a whole.

In contrast to the dominance of cancer in terms of disease burden and the high proportion of cancers attributable to modifiable factors, the majority of cancer research investment continues to be made in basic science. Also, clinical translational research has been foccused on the development of new therapies or aleatory trying to improve efficacy of previous treatments. On the contrary,

investment in primary prevention has often been neglected, partly because the results are difficult to recognize and its impact may take several decades to emerge (Wild, Espina et al. 2019). Despite of the molecular and genetic alterations that are of course very important for the undersanding of the disease, they must be integrated in a complex context of adaptation, and realize that when a single pathway is blocked, the WOB adapts and other pathways are activated, to try to compensate the blockage. This is probably the main reason why cancer treatments often fail and neoplastic cells become insensitive to therapies in a way that, when the disease is apparently controlled, cancer cells return with an even more aggressive pattern. Investigation should be foccused on “from macro to micro” along with “from micro to macro”.

The first question that I asked, had to do with the reality of neuroendocrine neoplasia in Portugal, as the figures previously available were based on studies conducted in other coutries. Since the formation of ENETS in 2004, many associations of NEN were constituted all over the world. In 2009 SPEDM, at that time chaired by Professora Manuela Carvalheiro envisaged to create GE-TNE, which I had the honour to chair. From the beginning, one of the main objective of the group, was to create a Portuguese Registry of Neuroendocrine Neoplasia. Since a prospective registry seemed too ambitious as an initial goal, a cross-sectional study enrolling the patients diagnosed with neuroendocrine neoplasia, attended at the main Portuguese Hospitals for a limited period of time seemed a satisfactory starting point. Besides, the group could test itself about the capacity of embarking on a broader project. So, SPEDM under the chair of Prof. Helena Cardoso, undertook the task to perform a study that lasted for 18 monthes and involved 293 patients from 15 health terciary centers. The results of the study were finally published in 2019 (Chapter 3.1) and provided a valuable insight into the epidemiology, current clinical practice and therapy strategies of neuroendocrine neoplasia in our country. Although slightly distinct from studies performed in northen Europe, results were similar to those found in other southern European countries and also in China, specially concerning the distribution of primaries, with panNEN being more frequent than GI-NEN. One of the most relevant aspects we found, was the lack of information about functional and genetic characterization of NEN, specially panNEN in young adults, in whom

a precise characterization of the hormonal and genetic profile would be essencial in defining the strategy for the management plan. This finding highlighted the absolute need to concentrate the resources in a limited number of highly specialized reference centers with a skilled multidisciplinary team to define treatment strategies with the mandatory enrolement of an endocrinologist expert. Unlike other cancers, these tumors have a unique profile that involves not only debulking and targeting the tumor mass, but also the need to evaluate and manage hormone secretory pattern, whose knowledge could make the difference in defining treatment strategy plan. For instance, the quality of life and prognosis of serotonin secreting midgut tumors, depends not only from the tumor clinical behaviour, since these are slowly growing tumors, but also from the control of secretory diarrea and carcinoid heart disease. Not by chance, the introduction of SA for controlling carcinoid syndrome symptoms increased 5-year survival from 18% to 67% (Anthony, Martin et al. 1996) by controlling flushing and diarrea in 36-100% of the patients (Modlin, Latich et al. 2006). Although neuroendocrine neoplasia are rare malignancies that tend to be neglected because the number of affected patients is low, having a dedicated team with expertise in treatment, research and teaching of these rare condition is undoubtly of added value for institutions seeking to reach excellence in healthcare services.

Another added value of GE-TNE study was that it allowed to set the ground for the development of a National Registry of NEN. It reinforced the need for a national clinical framework for GEP-NEN, in order to ensure a systematic surveillance of the disease and ultimately improve the diagnosis, clinical management and outcome of NEN patients. An important clue, is that the study opened the door for exporting our data into the ENETS Registry, the European Neuroendocrine Tumor Registry endorsed by ENETS, in which meetings GE-TNE group members and myself had the opportunity to participate. These last two projects are the current objectives which the actual study group chair is commited to complete, which I hope will allow Portuguese GE-TNE to have international projection behond the already consensual national visibility.

The next step of our work was to test the hypothesis that NEN are associated with modifiable RFs like obesity, MetS and T2-DM, already stated for some non-neuroendocrine cancers (Chapter 3.2). Globally, our findings support that, as

what happens with other non-neuroendocrine malignancies, there is an association of WD GEP-NEN with MetS. I believe this is one of the first studies performed in a cohort that includes almost all primary sites and stages as well as sporadic functioning and non-functioning, and also G1 and G2 tumors. The other two studies published about this subject were performed in rectal NEN detected accidentally by colonoscopy. The reason for choosing only WD GEP-NENs for the present work, is that it is consensual that these are distinct from NEC in terms of clinical behaviour and physiopathology (Heetfeld, Chougnet et al. 2015). Also, my personal clinical experience of the past 25 years enabled to observe that patients with WD NEN often present obesity, while in patients with NEC, as well as progressive undifferentiating WD NEN, whom weight loss and cachexia predominates. I think there are also major differences between WD NEN and NEC, from a metabolic perspective. In fact, functional nuclear medicine imaging shows that WD GEP-NENs express mainly somatostatin receptors on ^{68}Ga-PET-SSTR and are negative on ^{18}F-FDG-PET, while the NEC pattern is the inverse. In between, a progressive transition from WD NEN to NEC with a progressive loss of expression of somatostatin receptors and increasing captation of ^{18}F-fluorodeoxyglucose can be found in NEN with intermediate features (Hofman and Hicks 2012). In fact, diabetes was associated with advanced stages and more aggressive tumors in previous studies (Valente, Hayes et al. 2017).

This hypothesis for the disease mechanisms underlying NEN progression shares some similarities with T2-DM pathophysiology. At one extreme, in an earlier phase, glucose intolerance caused by IR associated adiposity dominates and is compensated by hyperinsulinism to maintain normal fasting glucose. On the other extreme of the spectrum, there is insulin deficiency caused by pancreatic beta-cell exaution and declared hyperglycemia along with weight loss and even cachexia (Leahy 2008). In between, pancreas begin to loose its compensatory capacity, fails compensatory insulin hypersecretion and beta-cells become exhausted. Hyperglycemia manifests at the same time and the patient begin to loose weight. In a similar way, obesity-related cancers such as breast, endometrium and colorectal are accompanied by obesity and IR at earlier stages, but as illness progresses, weight loss and a state of caquexia get installed and ultimately the patient perishes.

Following this line of investigation, the next step of our work was to evaluate whether the presence of MetS and individual MetS components at the time of WD GEP-NEN diagnosis was associated with any specific tumor characteristics, namely primary site, hormonal secreting syndrome, WHO grading and tumor extension (Chapter 3.3). We divided our patient cohort into two groups, with MetS and without MetS, as well as the individual MetS criteria, which were then compared. Our results demonstrate, for the first time to our knowledge, a positive association of MetS with WD GEP-NEN tumor grade and disease extension. Patients with WD GEP-NEN and MetS are more likely to have tumors with better differentiation and disseminated disease at diagnosis, independent of PT location and hormonal status. Contrary to what is commonly accepted, we did not find any association of FPG with pancreatic primaries (Capurso, Falconi et al. 2009) or with secreting tumors (Capurso, Falconi et al. 2009, Gallo, Ruggeri et al. 2018). The results obtained corroborate the hypothesis that better differentiation is linked to obesity and IR. We also found that metabolic abnormalities were associated with advanced disease, which could be explained by the fact that even GEP-NEN with low grading have already distant metastasis at presentation (Kloppel 2011).

Although as a clinician I focused most of my research in the clinic, I was also interested in investigate whether our clinical findings had some translation in terms of molecular profiling (“from macro to micro”). So, our last step was to investigate the influence of MetS and individual components in the molecular inflammatory and metabolic profile of WD GEP-NEN by analysing markers in tumor free surrounding tissue of the surgical specimen (Chapter 3.4). We found that IL-6 expression, a marker of inflammation in tissues surrounding GI-NEN was influenced by MetS features and positively associated with disease progression, suggesting an association between metabolic clinical features and an inflammatory status at cellular level. These results favour the hypothesis that similary to other cancers, WD-GEP-NEN may emerge from an inflammatory milieu caused by a pathological context of IR and MetS with an impact on prognosis.

Overall, from my personal among other authors perspective, there is a link between obesity, IR, expression of somatostatin receptors and WD GEP-NEN, which respond very well to treatment with SA (Rinke, Muller et al. 2009, Caplin, Pavel et al. 2014). On the other side of the spectrum, NEC are associated with high glucose uptake on ^{18}F-FDG-PET, cachexia, and lower response to somatostatin receptor inhibition. Although the national survey presented at Chapter 3.1 was not designed for this specific objective, data obtained corroborates this hypothesis, as patients with well differentiated NEN presented a significantly higher mean BMI (p=0.015) in comparison with NEC patients. Results from the cross-sectional Portuguese study also show that NEC patients had less metabolic co-morbidities than WD NEN. A brief analysis of patients co-morbidities revealed that high BP and T2-DM were more frequent in WD GEP-NEN than in NEC (16.3% vs. 11.1% for hypertension and 10.1% vs. 3.7% for T2-DM), while dyslipidaemia was more frequent in NEC (8.5% vs.11.1% for dyslipidaemia).

My personal believe is that for any reason, during the natural history of the disease, there is a downregulation of somatostatin receptor expression and GEP-NEN become resistant to somatostatin analogues, along with a downstream shift in neuroendocrine cells metabolism towards a more aggressive pattern of proliferation that is traduced on functional images by an increase captation on ^{18}F-FDG-PET. In the same way, mTor inibitors and tyrosine kinase inhibitors like sunitinib showed to have timelimiting anti-proliferative effect on WD panNEN (Barbieri, Albertelli et al. 2014). The results obtained on Chapter 3.4 corroborate this hypothesis as Ki-67 (a marker of proliferation) and FOXM1, which is activated downstream in Pi3K/Akt/mTor pathway, were found to be positively correlated both in panNEN and in GI-NEN. In addition, a statistically significantly positive correlation between IGF1R and FOXM1 in GI-NEN was found.This is consistent with the findings that resistance to mTor inhibition is due to a feed-back activation of Akt through a IGF1R dependent mechanism previously described (Wan, Harkavy et al. 2007). FOX01 is one of the downstream products of Akt that stimulates cell cycle activation and cell proliferation (Wan, Harkavy et al. 2007). So, its correlation with Ki-67 proliferating index would not be a surprise.

Although all these findings need to be validated by larger multicentic and prospective studies, they open a window for better understanding the actual GEP-NEN`s burden and disclose new lines of research concerning disease prevention and treatment. *In vitro* experiments demonstrated the anti-proliferative effect of aspirin in human pancreatic, bronchopulmonary and midgut neuroendocrine tumor cells (Spampatti, Vlotides et al. 2014). Even though in vitro action of metformin in NEN has been described in 2014 (Vlotides, Tanyeri et al. 2014), recent papers suggest an important role of metformin as adjuvant treatment of NEN`s conventional therapy (Pusceddu, Buzzoni et al. 2016, Thakur, Daley et al. 2019). The ongoing MetNET-2 Trial, was designed for the evaluation of the association of Lanreotide Autogel® and metformin in the treatment of advanced gastrointestinal and lung neuroendocrine neoplasia (Pusceddu, Prinzi et al. 2017). As previously mentioned in Chapter 1.5.2, a 2019 spanish publication (Herrera-Martinez, Pedraza-Arevalo et al. 2019) demonstrated that metformin and statins can exert a inhibitory effect of cell proliferation in either GI, pancreatic and lung neuroendocrine cell lines and that SSTR expression in lung carcinoids was higher in patients with diabetes treated with metformin than in those untreated. These conclusions also support the hypothesis that better differenciated tumors, could be the more insulin resistant, as those treated with metformin have more SSTR expression.

Chapter 5 - LIMITATIONS AND FUTURE PERSPECTIVES

"… So do birds think, or so at least Mr. Palomar thinks. "Only after having known the surface of things," he concludes, can we venture to find what lies beneath. But the surface of things is inexhaustible."

Free translation from "Italo Calvino, Palomar, 1985"

The work described in the present dissertation is inedit in Portugal and somehow innovative in the scientific community, yet there are some limitations to be considered.

First of all, although the majority of cases published in Chapter 3.1 come from the largest terciary hospitals in the country, these are not necessarily representative of the whole population, as the cohort was not aleatorialy selected. The main problem we found in the study group was that, with the exception of few institutions, the majority of the hospitals patients are not under the care of a dedicated specialized team. So, patients are attended by several specialities and the data obtained refers solely to those patients to which the principal investigator had direct access. Second, the other three studies (Chapters 3.2, 3.3 and 3.4) were single centre studies centred at the Portuguese Institute of Oncology of Oporto, although this is the hospital that in Portugal concentrates more patients with neuroendocrine neoplasia. Even at IPOFG, Porto, many cases had to be excluded, because at the time of study enrolment were already under pharmacological treatment. This problem had to do with some difficulties in including the patients at the time of admission. In that way, an entirely prospective study was not feasible and data before treatment was retrieved from files of other healthcare institutions. Consequently we have some missing information, that however was limited to a very small number of cases.

Another limitation was the difficulty to have at the same time the pathology files of operated patients who have also the clinical and analytical evaluation needed to access the presence of MetS, which restricted the number of patients included in the work presented in Chapter 3.4. We then reinforce the need to concentrate the efforts at a nacional level to conduct multicentric research studies, which are essencial to study rare diseases in order to achieve meaningful progress and innovation.

Another problem we faced was the lack of published international literature about obesity, metabolic syndrome and IR in the field of GEP-NEN, so we could compare our results. On one side there are thousands of publications about obesity, diabetes and non-neuroendocrine malignancies, but on the other side, the number of scientific articles in the field of NEN is scarce.

When I have designed the hypothesis formulated at the present work, I imagined final conclusions would be the end of the investigation. To my surprise, our findings are only the beginning of a long road, as “the surface of things are inexhaustible” as it was written by Calvino. Thus, these lines are not definite statements, these lines just point out that the presented line of investigation makes sense, and that maybe it is worth to continue on trying to reach the “depth”, since at the same time our results have answered some questions but also raised many doubts. So, we concluded afterall that the work here presented was just preliminary, as there are many unanswered issues of which we stress:

- Does the presence of MetS or MetS individual components have an influence on overall survival and progression free survival of patients with GEP-NEN?
- Are gut hormones, such as CCK, GIP, GLP1 and GLP2 and PYY involved in the pathogenesis of NEN? If this is to be confirmed, in what way are these part of a feed-back mechanism in which the lack of a substance, peptide or hormone leads to compensatory secretion with further hyperstimulation of proliferation of neuroendocrine cells in a particular site, in a similar manner as observed in type 1 GEN?

- Is there any association between the presence of MetS or any MetS individual components with the captation pattern of both ^{68}Ga-PET-SSTR and ^{18}F-FDG-PET?
- What happens in terms of metabolism of neuroendocrine cancer cells that could explain the fact that after a more or less prolonged period of slow growing WD GEP-NEN, there is a turning point when the tumor cells achieve a more undifferenciated pattern and disease rapidly progresses becoming unresponsive to all the drugs approved for treating the disease?
- Is there any difference between WD GEP-NEN and NEC patients in terms of metabolic profile?
- What is the role of metformin and other drugs used for MetS in the prevention, prognosis and survival of patients?

If these and other premises are to be confirmed, a fastinating field of investigation could be openned.

Chapter 6 - CONCLUSIONS

Obesity, diabetes and cancer are major burdens that healthcare systems face nowadays, not only in terms of morbidity and mortality but also in terms of economic costs which in the long term can become unbearable for economies. Projections for 2030 are not optimistic, as new emerging countries are going to join or even surpass developed countries in terms of incidence and prevalence of these diseases with the aggravating problem of having less technical and human resources. If millions are invested on medical research in order to discover new more efficient and safer treatments, priority must also been given to find the epidemiological and pathophysiological mechanisms that are responsible for the major disease burden in our societies.

In the past a tumor was considered an inert volume of proliferating cells. Modern view of oncology considers neoplasia as a true new organ that has gained autonomy in order to survive, with multiple functions and interactions with its microenvironment. One could say metaphorically that obesity is a “tumor of the adipose tissue”. In the beginning, adipose tissue accumulation is reversible and is relatively easy to loose weight. On more advanced stages, a steady state is reached and the probabily of reversion becomes more limited, since adipose tissue continues to proliferate and does not obey to regulation of endocrine feed-back mechanisms, turning obesity so difficult to treat.

Similarly, in atherosclerosis the endothelium begins to multiplicate to form a plaque that continues to proliferate, invades the vessels wall and migrates away (“metastise”) to target organs such as the heart or the cerebrum, reinvade and grow in the new location. So, both at cellular level and macro level, cancer could ultimately be considered a metabolic disease. These findings are challenging oncology tradicional paradigms and are going to change oncologic treatments in a way that, along with anti-neoplasic terapy, it will be possible to intervene in cancer in a similar way of metabolic diseases: not only by restricting energy at

dietary level and physical exercise promotion but also by chemoprevention, acting on the same targets of metabolic diseases, with metformin, PPAR antagonists, statins and other new drugs. Prevention will be of utmost importance in the triangle that constitutes priorities for cancer management, along with treatment and screening.

As final conclusion, I may say that investigation of the connection between very prevalent diseases and a rare disease like NEN, would be a challenge for the future, but the rationale for that line of investigation somehow makes all sense. Multicentric prospective studies envolving a large number of patients are needed for validating (or not) our results. If these finding are to be confirmed, a fastinating research line could be open as the world of IR, digestive polipeptides and NEN is still a field waiting to be explored.

Chapter 7 - REFERENCES

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