UNIVERSITÀ
DEGLI STUDI DI TRIESTE

DIPARTIMENTO
DI SCIENZE DELLA VITA

UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXXI CICLO DEL DOTTORATO DI RICERCA IN BIOMEDICINA MOLECOLARE

STARD3 and the identification of new cholesterol transport inhibitor

DOTTORANDA
BARBARA SALIS

COORDINATORE
PROF.GERMANA MERONI

SUPERVISORE DI TESI
PROF. GABRIELE GRASSI

CO-SUPERVISORE DI TESI
PROF. FLAVIO RIZZOLIO

ANNO ACCADEMICO 2017/2018

INDICE

ABSTRACT

Although many advances in the cancer treatment have been made, the research is continuously searching new perspectives in order to provide the best possible outcome for all patients. An emerging challenge is the identification of new genes involved in cancer development and progression in order to develop novel therapeutic molecules to use alone or in combination with current therapies. In the last years, some research groups have focused their attention on a protein initially discovered to be overexpressed in breast cancer samples: the StAR-related lipid transfer domain-3 (STARD3). STARD3 is a member of a subfamily of lipid trafficking proteins characterized by a C-terminal steroidogenic acute regulatory domain (STARD), which shares a 35% of homology with the domain of StAR protein, STARD1, a transporter of cholesterol in mitochondria. They both belong to the START (steroidogenic acute regulatory protein–related lipid transfer) proteins family, involved in the non-vesicular transport of lipids in membranes. The crystal structure of the START domain of STARD3 revealed a hydrophobic cavity formed by the α/β helix grip structure of the 210 amino acids with which it binds one molecule of cholesterol at an equimolar ratio 1:1, transporting sterol from the endoplasmic reticulum (ER) to the endosomes. In human, it was demonstrated that STARD3 is overexpressed in different cancer cell lines and, in particular, in Her2 overexpressing breast cancer. In fact, STARD3 and HER2 are co-amplified and co-overexpressed in about 25% of breast cancers. The molecular mechanism by which STARD3 cooperates with others oncogene such as HER2 is still unclear.

Nevertheless, STARD3 is implicated in therapy resistance of breast cancer, moreover, patients with a high level of STARD3 expression display metastasis, local recurrence and shorter overall survival. Recently, new evidences suggested a possible STARD3 overexpression also in colorectal, prostate and gastric cancers. Due to its involvement in cancer, STARD3 represents an attractive candidate as a target to cancer therapy and the

identification of selective inhibitors is an undiscovered but interesting field of study. In collaboration with the University of Pisa that has developed the first pharmacophore-based virtual screening (VS) platform focuses on the identification of new inhibitors of the STARD3 mediated cholesterol transport, we carried out a study to identify a lead compound (VS1) endowed with an interesting activity, thus representing the first reported STARD3 inhibitor. The activity of the inhibitor was evaluated in breast and colorectal cancer cell lines by analyzing cell vitality and the level of focal adhesion kinase (FAK). Inhibition of STARD3 by VS1 results in a consistent reduction of cell vitality; additionally the activation of a specific STARD3 target (FAK) produced by the ligand, suggests a potent and specific activity of VS1 at cellular level.

1. INTRODUCTION

1.1 COLORECTAL CANCER

Colorectal cancer (CRC) is the fourth most commonly diagnosed cancer and the second one for mortality (Bray et al, 2018). Incidence and mortality rates are considerably higher in males than in females (Siegel et al., 2015). CRC is a very heterogeneous disease caused by the interaction of genetic and environmental factors. There are different grade of the disease in which accumulation of genetic and epigenetic mutations are responsible of the transformation of the normal colonic mucosa into neoplastic tissue.

The World Health Organization (WHO) determines an increase of 77% in new diagnosed cases of CRC. Although these statistics the incidence of CRC did not increase in the past few decades, and the mortality rate decrease. This improvement is due to new better therapeutic regiments and preventive screening that were able to reduce the death, in particular in the early stages (Howlander et al., 2014). The main therapy for colorectal cancer remains surgery and at the early stages and with low risk, local resection can be resolved. On the other hand, chemotherapy after the surgery could improve the survival of the patient at the medium and late stages of the disease.

Colorectal cancer develops through progressive abnormal growth of the colon epithelium, which over time can transform to an adenomatous polyp and then cancer (Fearon et al., 1900). The majority of CRC arises through transformation of an adenomatous polyp, but only 5% of those polyps progress to cancer (Church JM et al., 2004). Being a multifactorial disease, in the malignant transformation of CRC the development and progression is due to different interactions between somatic and germline genetic, transcriptional, epigenetic and other regulatory events. Genetic alterations involve mutations in adenomatous polyposis coli (APC) during earlier stages and mutations in rat sarcoma viral oncogene homolog (RAS) and tumor protein P53 (TP53) during later stages (Rodrigues et al., 1990; Fodde et al., 2001; Lievre et

al., 2006). But only in the 7% of CRC cases has been found the combination of these three genes, underling the importance of other genes in the tumorigenic process (Smith et al., 2002). Epigenetic alterations in CRC occur early and manifest more frequently than genetic alterations. Advances in genomic technologies have led to the identification of a variety of specific epigenetic alterations as potential clinical biomarkers for CRC patients.

The median age at time of diagnosis is about 70 years. The incidence rate of CRC is different in the vary population worldwide, especially the incidence rate is 10-fold higher in the US and Europe than in African and Asian countries. The incidence rate of immigrants change with the generations: the first generation is almost similar of their home country, but from the second one the incidence rates adapts to the immigration country (Stintzing S. 2014). The wide geographical variation in incidence rates for colorectal cancer, and data from migrant studies, suggest that lifestyle risk factors, including diet, physical activity, obesity and diabetes, play an important role in the etiology of the disease. In fact, a high consumption of different processed foods and alcohol has been associated with higher colorectal cancer risk (Huxley et al., 2009). However, enormous disparities in colorectal cancer survival exist globally and even within regions due to differences in access to diagnostic and treatment services (Boyle and Langman, 2000). Patients with inflammatory bowel disease also have a higher risk of CRC and they are undergone to surveillance programs. Genetic and epigenetic elements can be considered as risk factors, since they increase predisposition to CRC and are currently used as biomarkers, for instance, to predict progression or resistance to specific therapies (Prenen et al., 2013).

Primary prevention strategies for CRC sporadic cases includes first of all particular attention to eating habits with an increase in whole grains, fruits and vegetables, and practice physical activity. In the other hand, reinforcing the screening programs in some countries has led to detection of CRC in the earlier stages. In combination with the development of more effective treatment options for the metastatic stage, 5-year survival rates have improved throughout all

stages, from about 51% in the ’70s to about 65% in the 2000s when all races are taken together (Siegel RL et al., 2015).

1.1.1 CLASSIFICATION

Over the past few years, is well established that CRC is a very heterogeneous disease and the prognosis and the good therapy response are absolutely dependent from molecular and genetic elements (Prenen et al., 2013).

The vast majority of cancers are a result of sporadic genetic events; only rare cases (<1%) have an inherited component. The accumulation of multiple mutations in the sporadic cases gave advantage in cancer growth, invasion, and metastasis. While “gain of function” mutations occur in oncogenes such as KRAS , many of the genetic events that underlie cancer seem to be inactivating, or “loss of function,” mutations affecting tumor suppressor genes in the case of APC gene and the consequently downstream pathway.

For a better understanding and study of the disease is necessary an appropriate classification of the different stages and causes involved (Figure 1). The most commonly used colorectal cancer staging system is the TNM system, established by the American Joint Committee on Cancer. The TNM staging system looks at three key factors to determine the stage of cancer: the size of the primary tumor (T stage), the involvement of lymph nodes (N stage), and the occurrence of distant metastases (M stage) (Hamilton, S.R. et al., 2000). In the progress of the disease there are different stages from the early (stage 0) to the metastatic state (stage IV). In the earliest stage the cancer has not grown beyond the inner of the colon or rectum. Patients with stage I and II (Union for International Cancer Control) are not treated with adjuvant chemotherapy after surgery, showing a good 5-year survival rate. In stage III, in which primary tumor stated to metastasize to the local lymph nodes are treated with adjuvant

treatment after surgery in order to reduce a relapse after surgery. In stage IV, in metastatic state, the survival rate remains at about 15% (Kopetz S. et al., 2009).

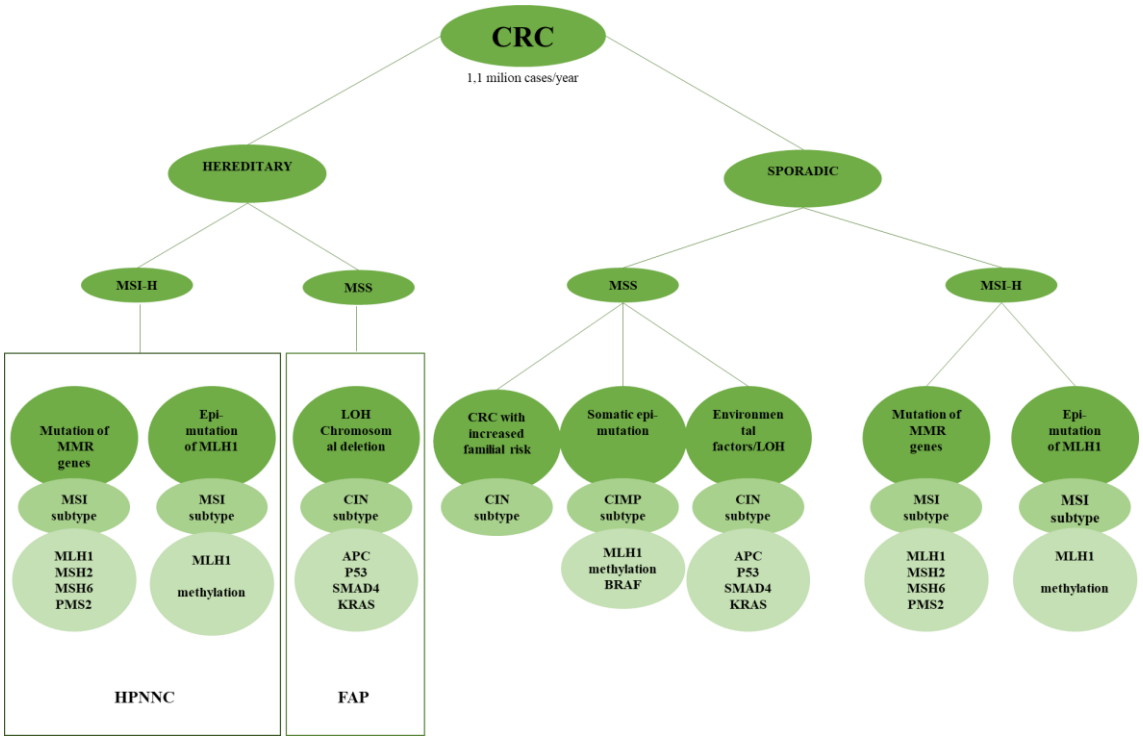


Figure 1. Molecular classification of colorectal cancer. Colorectal cancer could be primarily divided in inherited and in sporadic. Adapted from Fleming et al., 2012.

As preview reported, the majority of CRCs are sporadic (70%-80%), with age being the most important risk factor. Several progress have been made in understanding the molecular basis of sporadic colon cancer.

Only a small proportion of cases are due to inherited forms, the most known syndromes are familial adenomatous polyposis, FAP (less than 1%), non-polyposis hereditary CRC or Lynch syndrome (2%-5%) and *MYH*-gene associated polyposis (< 1%). An additional 20%-25% of cases are estimated to have an associated hereditary component, which has not yet been well established and is known as familial CRC (Farrington et al., 2005; Piñol V. et al., 2005).

Patients with FAP develop large number of benign adenomatous polyps of the colorectal epithelium in their early adulthood, some of these will progress with invasiveness and metastasize. Colorectal cancer occurs in Lynch syndrome patients at an average of 45 years and an increased risk of malignant development in extra colonic organs may arise (Lynch et al., 2003).

1.1.2 MOLECULAR CARCINOGENESIS AND CLASSIFICATION OF COLORECTAL CANCER

Vogelstein and colleagues proposed the accumulation of genetic alterations, in particular APC, TP53, and KRAS mutations, to be responsible for CRC development (Vogelstein et al., 1988) (Figure 2).

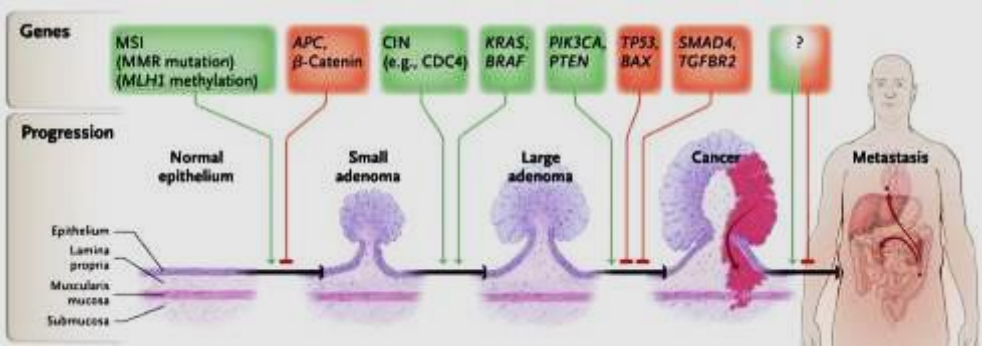


Figure 2. Genes and growth factor pathways that drive the progression of colorectal cancer. Adapted from Markowitz and Bertagnolli, 2009.

Genomic instability is a fundamental process in colorectal carcinogenesis (Beckman 2005). In sporadic cases there are three major distinct tumorigenesis pathway. The 15% are characterized by microsatellite instability (MSI) due to aberrant mismatch repair machinery and the chromosomal instability (CIN) phenotype, which accounts for 70%, exhibits gross

chromosomal abnormalities such as aneuploidy and loss of heterozygosity (LOH) at tumor suppressor gene loci and chromosomal rearrangements (Grady, 2008). APC or β-catenin mutations are the most common initial molecular lesion in CIN phenotype. The third pathway, designated as CIMP, is characterized by a widespread CpG island methylation (Toyota et al., 1999). Approximately 30-40% of sporadic CRC are CIMP- positive (Hawkins et al., 2002).

The accumulation of mutations due to chromosomal instability arise in oncogene activation of KRAS and suppressor gene inactivation (APC, SMAD4 and TP53) (Fearon and Vogelstein 1990). The overwhelming number of the sporadic cases implied abnormalities in WNT pathway with a hyperactivation due to mutations in APC gene. In fact, over 80% of adenomas and CRC has APC mutations and 5-10% shows mutation in β-catenin and other components of WNT signaling resulting in hyperactivation of WNT pathway (Silva AL et al., 2014). APC is an important negative regulator of WNT pathway, promoting the proteosomal degradation of β-catenin. When APC degradasome complex presents a mutational inactivation of APC, β-catenin accumulates in the cytoplasm and translocates in the nucleus, with the consequence of an activation of MYC and other genes (Bienz M. et al., 2000). Consequently to a dysregulation of the WNT pathway there is a consistent differentiation of the colon epithelium, with the development of dysplastic crypts. With ulterior mutations in tumor suppressor genes, such as TP53, usually there is a transition from adenoma to invasive carcinoma In the hereditary cases the Lynch syndrome plays an important role. This disease is an inheriting susceptibility to cancer of several organs, including colon. The neoplasm is due to inherited defects in the DNA mismatch repair in neoplastic cells, in which MMR gene is mutated. All somatic cells contain one wild type and one mutated MMR allele. But during tumor formation, MMR allele is inactivated and neoplastic cells have both MMR alleles inactivated. This results in accumulation of mutations in coding and non-coding microsatellites, so-called microsatellite instability (MSI).

1.1.2.1 CHROMOSOMAL INSTABILITY PATHWAY-CIN

The chromosomal instability (CIN) pathway is the most common pathway in the leading to CRC reaching the 75-80% arising from adenoma precursor lesions (Cancer Genome Atlas Network et al., 2012). This model is linked with accumulation on numerical or structural chromosomal abnormalities, and is characterized by frequent loss of heterozygosity at tumor suppressor gene loci, but the causes of CIN in CRCs and its underlying mechanisms remain unknown (Pino et al., 2010). In the CRC tumorigenesis, several accumulation of mutation in specific oncogenes (e.g. APC, KRAS, BRAF, SMAD4, TP53, etc.) and tumor suppressor genes are involved. An important initiating mutation for early adenoma development is the mutation of the adenomatous polyposis coli (APC) tumor suppressor gene, which has a role in the WNT pathway (Steven M. Powell et al., 1992)

1.1.2.2 MICROSATELLITE INSTABILITY PATHWAY- MSI

The second major colorectal neoplasia pathway in which adenomas are involved is the microsatellite instability (MSI) pathway. Around 15% of CRCs possess genetic instability due to MSI (Jeremy R Jass et al., 2006). Of these tumors, approximately 20% have a hereditary cause and arise in patients with a germline mutation in one of the mismatch repair genes, the Lynch syndrome (Stephanie A Cohen et al., 2014). The other 80% of MSI CRCs seem to arise due to the hypermethylation of the promoter of the human MutL homologue (hMLH) 1, a specific mismatch repair gene MLH1 (Richard Boland et al., 2010). MSI tumors are distinguished by dysfunction of DNA MMR genes leading to genetic hypermutability. Deficiency in DNA repair as a consequence MMR silencing genes, generates several abnormalities in short sequences that are repeated up to hundreds of times within the genome (microsatellites). MMR dysfunction will result in instability in the length of microsatellites, causing frame-shift mutations (Pérez-Cabornero et al., 2011). Typical frameshift mutations

are found in specific genes such as β-catenin, transforming growth factor β receptor II (TGFβRII), epidermal growth factor receptor (EGFR) or Bcl-2-associated X protein (BAX) (Kim et al., 2003; Fernandez-Peralta et al., 2005; Liefers et al., 2002). MSI CRCs are enriched for the epigenetic inactivation of the MLH1 gene, being CIMP positive. These phenotypes show high frequency of the BRAF mutation and a low frequency of APC and TP53 mutations. Compared with the CIN pathway, WNT pathway activation is more often due to a mutation in β-catenin than in the APC gene, in fact he WNT signaling pathway is believed to play a gatekeeper role in both CRCs (Miyaki M et al., 1999; Cancer Genome Atlas Network, 2012). Alterations in the MAPK pathway seem to play a role in CRC development, in addition KRAS is often mutated in these patients (C. Richard Boland et al., 2010).

The phenotype is characterized by their occurrence in females at a late age and by right-sided location, mucinous cell type with poor tumor differentiation and presence of tumor infiltrating lymphocytes.

Recent studies suggest that MSI is a possible marker of good sensitivity to therapy with 5-fluorouracil (5-FU) particularly in the presence of deletion in HSPH1 (Collura et al., 2014; Dorard et al., 2011). The responsiveness to 5-FU in MSI CRCs is strongly associated with the stages of the disease. In MSI tumors at stage II the sensitivity to 5-FU lack, instead in stage III is controversial and further studies are required (Kawakami et al., 2015; Vilar et al., 2010; Sargent et al., 2010; Gavin et al., 2013).

1.1.2.3 CpG ISLAND METHYLATION PATHWAY- CIMP

In addition to these genetic alterations, the presence of widespread CpG island methylation in CRC, leads us to the third pathway, which is designated the CpG Island Methylation Pathway (CIMP). Whereas CpG dinucleotides are underrepresented in the mammalian genome, approximately half of all human genes contain a CpG-rich region called a ‘‘CpG island’’ in

the 5' area, often encompassing the promoter and transcription start site of the associated gene (Bock et al., 2007). Gene silencing by hypermethylation of CpG islands (including tumor-suppressor genes) is a common event in tumors. It has been reported that CIMP positive tumors can be divided in two types, namely CIMP high, related to BRAF mutations and MLH1 methylation and CIMP low, related to KRAS mutations (Shen et al., 2007).

Interesting, CIMP status did not show any relationship with CRC prognosis. However, when CIMP profile is associated with MSI or BRAF mutations the CRC survival is altered (Jia et al., 2016).

In conclusion, although 70% of CRC arise via the well characterized chromosomal instability pathway, it seems that approximately 30 % of CRC develops via a serrated pathway which is characterized by activation of the MAPK pathway (KRAS or BRAF mutations, mutually exclusive) and the presence of CIMP (L or H) (Bettington M. et al., 2013).

1.1.2.4 POLE MUTATIONS

POLE mutations were identified in ultramutated CRCs in the TCGA study (Cancer Genome Atlas Network, 2012). POLE encodes a polymerase responsible for replicating nuclear DNA and that is involved, with other two polymerases (POLA1 and POLD1) in the synthesis stage of the DNA repair process (Popanda et al., 2000). The tumorigenesis importance of this polymerase has been demonstrated in endometrial and in colorectal cancer (Briggs et al., 2013). Germline mutations of POLE are predicted to cause an effect in the correction of the bases insertion during DNA replication, these tumors show MSS phenotype (Gong et al., 2017).

1.1.2.5 LINE-1 HYPOMETHYLATION

Genomic DNA hypomethylation can exert an influence on carcinogenesis influencing chromosomal stability, and consequently in the increasing of mutation rates (Chen et al., 1998). The reduction in methylation content is accompanied by repetitive transposable DNA elements such as the long interspersed nucleotide element-1 (LINE-1) or short interspersed nucleotide element. LINE-1 constitutes a substantial portion of the human genome, for about 18% (Rodic et al., 2013). With the imminent activation of LINE-1 retrotransposons the degree of chromosomal instability is higher, lead to transcription of adjacent genes and generation of gene transcripts involved in the regulation of gene expression or telomere maintenance (Faulkner et al., 2009; Mueller et al., 2018).

In CRC, LINE-1 hypomethylation phenotype is characterized by early age onset, MSS and CIN, and the presence of a CIMP-negative, BRAF wild-type phenotype (Baba et al., 2010). LINE-1 hypomethylation status of circulating cell-free DNA in plasma could be used as a potential biomarker for identifying aggressive CRC, particularly for the early stage form associated with a favorable prognosis (Inamura et al., 2014; Nagai et al., 2017)

1.1.2.6 CONSENSUS ON COLORECTAL CANCER SUBTYPE CLASSIFICATION

In the landmark publication of The Cancer Genome Atlas Network in 2012 (Comprehensive molecular characterization of human colon and rectal cancer) applying next-generation sequencing technique on 97 colorectal tumors, it has become clear that CRC is made up of a complex network of genetic alterations leading to the dysregulation of multiple pathways. At the 2014 American Society of Clinical Oncology Annual Meeting, four major molecularly distinguishable subtypes of CRC were proposed by the CRC subtyping consortium (Colorectal Cancer Subtyping Consortium (CRCSC) identification of a consensus Of molecular subtypes).

Colorectal cancer can be classified into four groups called consensus molecular subtypes (CMS1–4), each of which has a unique biology and gene expression pattern. The first one (CMS1 ~14%) are also known as serrated polyps. These lesions are enriched for MSI that display immune pathway activation, it affects especially females, in older age; high BRAF mutation rate, hypermethylation of CpG island, causing loss of tumor suppressor function (Thanki et al., 2017).

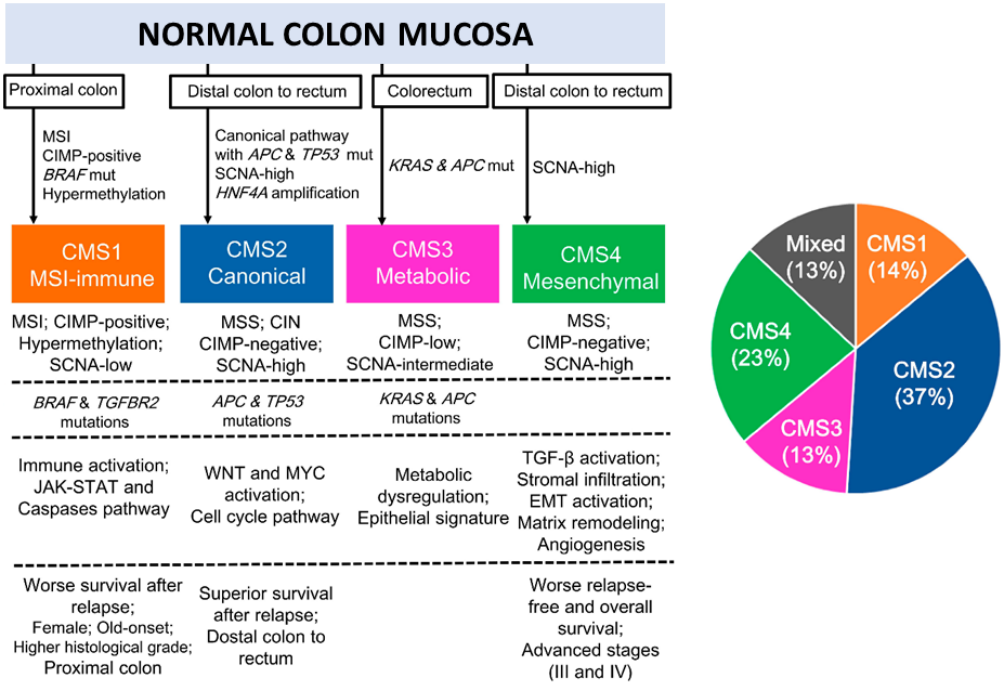


Figure 3 The taxonomy of colorectal cancer according to the Colorectal Cancer Subtyping Consortium, reflecting biological differences in the gene expression-based molecular subtypes (Guinney et al., 2015). Adapted from Inamura, 2018

CRC in the CMS2 category (~37%) arise from the canonical adenoma-to-carcinoma sequence. This profile is characterized by the initial loss of tumor suppressor gene APC, TP53 and activating mutation in KRAS. Therefore, CIN degree is high, MSS, displays epithelial markers (Dienstmann et al., 2017). CMS2 tumors have activated WNT-β catenin and MYC pathway activation and amplification/up-regulation of common oncogenes, including EGFR.

CMS3 tumors (~13%) also known as the metabolic subtype, have an epithelial phenotype but with mixed CIN/MSI status, intermediate levels of hypermethylation, higher proportion of KRAS mutations (68%) and deregulation of multiple metabolic pathways.

CMS4 tumors (~23%) exhibits low level of hypermutation, CIN and MSS status, younger age at diagnosis, characterized by stromal invasion linked to TGF-beta signaling activation and angiogenesis. Therefore, it presents inflammatory microenvironment with prominent innate immune cells (Guinney et al., 2015). The remaining samples (~13%) did not have a consensus assignment, most likely representing mixed subtypes, intra-tumoral heterogeneity or poor quality of RNA. Importantly, the CMS have strong prognostic implications, with improved overall survival and survival after relapse for CMS2 group, significantly reduced survival after relapse in CMS1 group and dramatically increased risk of metastasis and CMS4 group have a poor prognosis with the worst 5-year overall survival (62%) and relapse-free survival (60%) of any molecular subtype (Guinney et al., 2015).

1.1.2.7 GENETIC PATHWAYS AND MORPHOLOGY

Consistent with genetic models, there appear to be at least three distinct clinicopathologic evolutional routes to sporadic CRCs (Jass 2007; Pancione et al., 2012). The first one is the traditional pathway which start from normal mucosa via tubular adenomas, with APC mutations, and results in typical CRC in the distal colon, with CIN and TP53 mutation. The second one is the serrated pathway, starting from normal mucosa via serrated adenomas, due to BRAF mutations and CIMP, resulting in colon cancer with good prognosis with MLH1 loss and MSI. The alternative pathway is the third, with poor prognosis arising from serrated adenomas with KRAS, BRAF and APC mutations and CIMP) (Pancione et al., 2012). Superficial-type colorectal tumors and de novo cancer have been identified. In these cases,

KRAS mutation was rare (Minamoto et al., 1994; Umetani et al., 2000), instead TP53 and APC were frequently mutated (Aoki et al., 1994) (Figure 4).

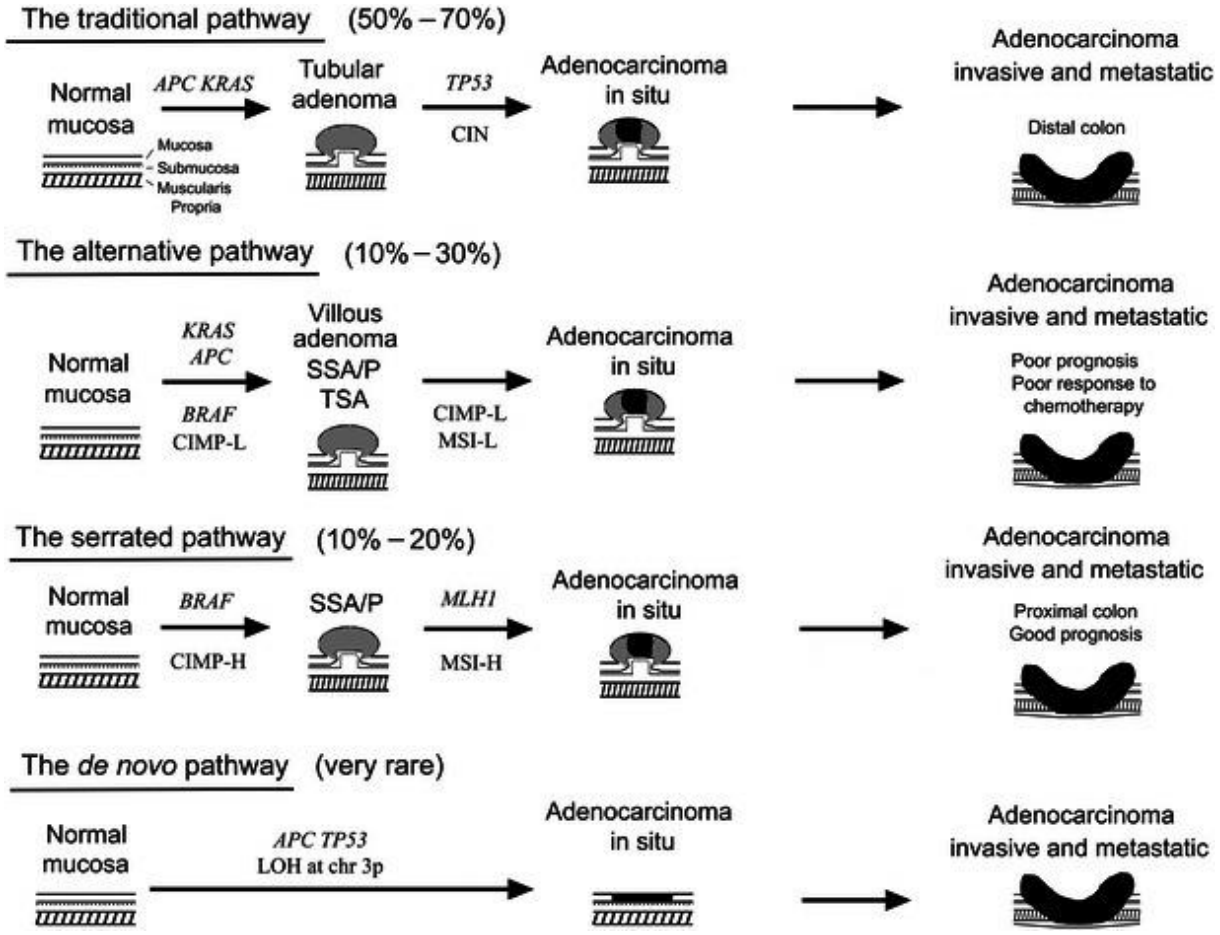


Figure 4 Evolutional pathways for colorectal morphogenesis. Adapted from Yamagishi et al., 2016

1.1.3 HEREDITARY COLORECTAL CANCER

The syndromes of CRC are defined on the basis of clinical, pathological, and more recently, genetic findings. Conditions that express adenomatous polyps include Lynch syndrome (also called hereditary nonpolyposis colorectal cancer [HNPCC]), familial adenomatous polyposis (FAP). All of these conditions are inherited, autosomal dominant disorders.

1.1.3.1 FAMILIAL ADENOMATOUS POLYPOSIS – FAP

FAP is characterized by hundreds to thousands of adenomatous colorectal polyps that develop in the second decade of life. It accounts for approximately 1% of CRC cases. FAP is inherited in an autosomal dominant matter by a germline mutation in the adenomatous polyposis coli (APC) gene.

1.1.3.2 HEREDITARY NON-POLYPOSIS COLORECTAL CANCER SYNDROME – HNPCC

Individuals with Lynch syndrome are predisposed to various types of cancers, especially colon and endometrial. HNPCC or Lynch syndrome is the most common inherited colon cancer syndrome, due to germline mutation in one of the different mismatch repair (MMR) genes. About 2-5% of all CRC cases are attributed to HNPCC.

1.1.4 PREDICTIVE TUMOR MARKERS

1.1.4.1 APC – WNT-β catenin- TGF-β pathway

APC was identified in 1991 by molecular cloning encoded by the FAP locus. Also sporadic colorectal tumors have both APC inactivated. Genetic disruption of APC, which leads to the activation of the WNT pathway, is a critical early event in colorectal tumorigenesis. The TCGA study reports that the WNT pathway was activated in 90% of CRC tumors (cancer Genome Atlas Network, 2012). In detail, 80% had APC mutations, the 5-10% exhibits

mutations o alterations in other WNT signaling components (cancer Genome Atlas Network, 2012; Vogelstein et al., 1988). It represents the most critical molecular target for the binding with β catenin.

In wild type conditions APC works as a negative regulator of the WNT pathway, promoting the degradation of β catenin, important activator of this pathway. Consequently, to APC mutations there is an accumulation of β catenin in the cytoplasm, that it translocates to the nucleus, arising in WNT signaling pathway dysregulation. The destabilization of this pathway results in transcription of downstream target genes with abnormal cell proliferation and differentiation of colonic epithelia, with adenomas, growing in high grade owing to the inactivation of other important tumor suppressor genes. In particular, with the inactivation of TP53 there is a transition from adenoma to invasive carcinoma (Thorstensen et al., 2005; Pino et al., 2010)

Mutations in β catenin with APC wild type, cause a resistance in Axin complex destruction. Furthermore, mutation in DNA mismatch repair deficient has intact APC (Satya Narayan et al., 2003).

In cellular processes such as cell growth, differentiation and apoptosis another pathway is protagonist, the TGF-β pathway, it serves as a tumor suppressor. Changement in the TGF-β contribute to the CIN pathway in CRC tumorigenesis. The main alteration is the loss of chromosomal 18q, that encodes for two important tumor suppressor genes, SMAD2 and SMAD4. The loss of which inactivates the pathway promoting apoptosis and cell proliferation. Even MYC became active by the TGF-β inactivation, indicating an important role in CRC. During the late stages this pathway switches to be oncogenic promoting invasion and tumor metastasis (Derynck et al., 2011; Xu et al., 2007; Katz et al., 2016)

1.1.4.2 TP53

Depending on the context, p53 can have different roles, inducing cell cycle arrest, apoptosis or senescence. Therefore in cellular stress conditions p53 can induce hypoxia, oncogene activation. Patients with mutation in p53 gene are often resistant to current therapies, arising in poor prognosis than those with wild type p53 (Iacopetta, 2003). p53 is a transcription factor that can be inducted by stress, it regulates several downstream genes to exert regulative function in multiple signaling processes. p53 mutations are find in approximately 40%-50% of sporadic CRC patients (Takayama et al., 2006). Different types of p53 mutations play a pivotal role in determining the biologic behavior of CRC, such as invasive depth, metastatic site and even the prognosis of patients. p53 mutations are associated with lymphatic invasion in proximal colon cancer, and show significant correlation with both lymphatic and vascular invasion in distal CRC (Russo et al., 2005).

Results from a large number of studies have unequivocally evidence demonstrated that mutant p53 not only plays a pivotal role in the transformation of CRC, but also contributes to the aggressiveness and invasiveness of CRC

1.1.4.3 MMR PATHWAY

One of the most studied genotypic subtypes of CRC is that characterized by a deficient mismatch repair (dMMR) pathway, usually found in combination with microsatellite instability (MSI). DNA replication is a process that may lead to the introduction of errors into new DNA synthesized or the presence of insertion/deletion loops. If they aren't unrepaired, these errors may result in permanent mutation that could be responsible for behavior changement of a cells. The cells have different repair mechanism including MMR pathway. Mismatch repair (MMR) proteins are nuclear enzymes, which participate in repair of base-base mismatch that occur during DNA replication in proliferating cells (Kheirelseid et al., 2013). The proteins bind to areas of abnormal DNA and initiates its removal. Loss of function

of this complex lead to an accumulation of DNA replication errors in particular area arising in the phenomena known as microsatellite instability (MSI) (Thibodeau et al., 1993).

Current estimates suggest that dMMR is present in 15–17% of all primary CRC (Imai et al., 2008; Popat et al., 2005). MMR genes usually function as a tumor suppressor gene, when both alleles loss function these genes loss the tumor suppressive effect. Alterations in at least six of the genes that encode proteins involved in the MMR system have been identified in either hereditary nonpolyposis colorectal cancer (HNPCC) or sporadic colon cancer. These genes include MSH2, MSH3, MSH6, MLH1, PMS1, and PMS2.

Inactivation of MSH2 or MLH1 generate MSI profile and HNPCC cancers frequently show loss of heterozygosity in these genes. Mutations that occurs in MSH2 and MLH1 can be missense, deletion or insertion. One wild type allele is sufficient to maintain MMR activity. While occasional somatic mutations of MSH2 and MLH1 were detected, the predominant mechanism for inactivating MMR unexpectedly proved to be the epigenetic silencing of the MLH1 promoter due to aberrant promoter methylation (Kane et al., 1997; Veigl et al., 1998).

dMMR CRC represents a distinct cancer subtype in terms of pathogenesis and this phenotype may also determine the response to therapy. Moreover, defective MMR cancers have shown an unclear and unpredictable response to DNA damaging agents, the elective therapy for CRCs: although not all studies agree (Aebi et al., 1997) most suggest that dMMR is associated with resistance to treatment at clinically achievable steady-state plasma concentrations of 5-FU (Remick et al., 1990).

In May 2017, the US Food and Drug Administration (FDA) granted the accelerated approval to pembrolizumab, a monoclonal anti-PD-1 (PDCD1) antibody, for patients with MSI or MMR-deficient solid tumors. This is the first time that FDA has approved a cancer treatment based on a common biomarker rather than an organ-based approach (American Association for Cancer Research, 2017).

1.1.4.4 EPIDERMAL GROWTH FACTOR RECEPTOR - EGFR PATHWAY

The epidermal growth factor receptor (EGFR), is a member of the human epidermal growth factor receptor HER-erbB family, is a transmembrane tyrosine kinase activated by the binding of extracellular ligands. These receptors are involved in several intracellular signals, responsible in the proliferation of cancer cells, differentiation, angiogenesis and metastasis dissemination. In particular, 60–80% of CRC patients with EGFR overexpression, this has been associated with poor prognosis and malignancy progression (Porru et al., 2018).

The EGFR pathway is indeed involved in colorectal carcinogenesis through the binding of EGF or other ligands on the extracellular part of the receptor, this activation results in the initiation of an oncogenic intracellular signalling cascade involving several pathways, including the RAS-mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt, phospholipase C, signal transducer and activator of transcription (STAT) and SRC/FAK pathways.

The importance of this pathway is highlighted not only by the identification of numerous alterations of its components, such as RAS, PI3K and BRAF, but also by the efficacy of the anti-EGFR therapies, in particular with cetuximab and panitumumab (Finnberg et al., 2015)

1.1.4.5 RAS

RAS isoforms are small molecules of 21 kD localized at the inner surface of the plasma membrane, which are coded by the three genes KRAS, NRAS and HRAS. They are GDP/GTP-binding proteins that act as intracellular signal transducers (Vetter and Wittinghofer, 2001) and the GTP-active form interact with a variety of downstream effector proteins (Marshall, 1996). The mutation in these families of genes cause RAS accumulation

in the active GTP-bound state (Bos, 1989). They are thus activating mutations of the RAS/MAPK pathway. The vast majority of these oncogenic RAS mutations in colorectal cancer affect amino-acid residues G12, G13 of the KRAS gene (Bamford et al., 2004). After binding and activation by GTP, RAS recruits the serine/threonine kinase RAF, which also acts as an oncogene that phosphorylates MAPK-1 and -2 and initiates MAPK signalling that leads to the expression of proteins having important role in cell proliferation, differentiation and cell survival.

KRAS is a proto-oncogene and KRAS mutations occur as an early event in about 50% of sporadic cases. Loss of APC or mutations in β catenin in mismatch repair deficient tumors depend on KRAS, but the degree of the connection is still under investigation (McCormick F., 2015). The prognostic relevance of KRAS mutations is controversial, in the majority of the studies KRAS mutation are associated with poor prognosis. Moreover, mutations in KRAS has been identified as a potent factor in the resistance to EGFR antibodies such as cetuximab or panitumumab (Heinemann et al., 2009), so patients with metastatic CRC and KRAS mutation in codon 12 or codon 13 are not recommended anti-EGFR therapy (Allegra et al., 2009).

1.1.4.6 BRAF

Another member of RAS family is BRAF, an oncogene mediator of the EGFR pathway. BRAF encodes a protein kinase involved in intracellular and cell division. The gene product is a downstream effector of KRAS within the RAS/RAF/MAPK cellular signaling pathway. Mutations in BRAF have been described in several cancer: 50-60% of melanoma have a constitutive activation of the BRAF kinase, thyroid are positive in 30-50% of the cases and in about 10% of colorectal cancer patients (Barras, 2015; Cheng et al., 2018). Triplet therapy

with the combination of anti–PD-1/PD-L1 therapy, BRAF, and MEK inhibitors have changed the treatment landscape in patients with BRAF-positive advanced melanoma, improving the patient outcomes (Luke J., et al., 2017).

In the early stage of CRC, BRAF mutations showing constitutive activation and deregulation of the downstream signaling pathway (Kim et al., 2014) resulting in uncontrolled, non-growth factor dependent cellular proliferation (Mojarad et al., 2013). A quasi-unique point mutation is observed in the BRAF gene, leading to the substitution of a valine by a glutamic acid in codon 600 (V600E) and is found in 10–15% of colorectal tumours (13%) (Bamford et al., 2004). Mutations in BRAF have been associated with poor clinical outcome (Ogino et al., 2009) and in combination with MSS tumours, patients have shorter progression-free survival and overall survival (Lievre et al., 2010). There are accumulating data showing that BRAF mutations are predictive of resistance to anti-EGFR therapy (Tol et al., 2009).

1.1.4.7 RAS, BRAF, AND PIK3CA MUTATIONS IN THE MAPK/PIK3 PATHWAY

MAPK and PIK3 (PI3K) pathways are both involved in cell proliferation (Mendoza et al., 2011). Alteration in these pathways contribute to providing advantage in proliferation for tumor cells. Mutations of KRAS, BRAF, and PIK3CA are the most common to affect the MAPK/PIK3 pathways in colorectal tumorigenesis. Approximately 40% of CRCs harbor KRAS mutations (Lee et al., 2017; Inamura et al., 2015; Jones et al 2017). recent studies suggest that BRAF and PIK3CA mutations also contribute to the resistance to anti-EGFR antibody therapy (De Roock et al., 2010; Van Brummelen et al., 2017; Xu et al., 2017). PIK3CA is an element in the PIK3 signaling pathway downstream of EGFR. Mutations in PIK3CA are present in 10–20% of CRCs and are associated with other altered phenotype such as KRAS mutant and CIMP-positive, and predict resistance to anti-EGFR antibody therapy

(Lee et al., 2017). In colorectal cancer, PIK3CA mutations occur more frequently in women, and in the proximal part of the colon (Benvenuti et al., 2008; Barault et al., 2008b), a double mutation of the gene is observed in 6–9% of the mutated cases (Samuels et al., 2004; Barault et al., 2008b) and there is a significant concomitant occurrence of KRAS and PIK3CA mutations (Parsons et al., 2005; Velho et al., 2005; Barault et al., 2008) In particular, these mutations active the PIK3 signaling pathway enhancing cell proliferation and in worst cases on carcinogenesis.

1.1.4.8 IMMUNE BIOMARKERS AND THE MICROBIOME

The impact of immune cells on tumor progression has been extensively reported in various cancer types, including CRCs (Tran et al., 2016; Kather et al., 2017; Boland et al., 2017).

Emerging evidence indicates that immune checkpoint mechanisms play a critical role in suppressing the anti-tumor T-cell-mediated immune response in the tumor microenvironment. CD274 (PD-L1) is an immune ligand repress antitumor immunity through the binding to PDCD1 (PD-1) receptor of T cells in various tumors (Masugi et al., 2016). Therapeutic antibodies that target PDCD1 and CD274 are effective in numerous malignancies, including CRCs (Overman et al., 2017).

In a recent study, inflammatory diets have been associated with a higher risk of colorectal cancer subtype with lymphocytic reaction. These finding suggested that diet related inflammation can promote the carcinogenesis by suppressing the adaptive anti- tumor immune response. The pro-inflammatory diet-associated CRC subtype was enriched in MSS, CIMP-low/negative, and BRAF wild-type phenotype (Liu et al., 2017).

These observations suggest that intestinal microbiota is a new player in CRC development. It has been proposed that CRC occurrence may also be influenced by the intestinal microbiota

which the gut is in constant exposition with. Several studies have linked a modification of intestinal mucosa-associated microbiota composition in patients with CRC compared to control subjects (Chen et al., 2012; Lu et al., 2016; Gao et al., 2015). It is well reported a model where the mechanism responsible of CRC influenced or induce by bacteria is based on the release of toxins produced by bacteria theyself, consequently the ambient is altered and there is a decrease of beneficial bacterial for the high levels of metabolites and the production of pro-carcinogenic compounds and alterations in the intestinal microbiota or dysbiosis. All of these mechanism can promote CRC development with an aberrant activation of the immune system and a chronic inflammation (Schwabe et al., 2013).

1.1.5 THERAPY

The treatment of CRC should be individualized and discussed in a multidisciplinary team. Surgery is the primary treatment for patients with potentially curable CRC. Depending on the stage of CRC, recurrence rates, survival times, and management are different.

The CRC Survival is tightly dependent on stage. In patients with CRC, 5-year survival is >90% for stage I, 60-85% for stage II, 45-65% for stage III and 5-7% for stage IV. But unlucky, at the time of diagnosis, about 20-25% of CRC patients have metastatic disease or will develop metastasis later (Schmoll et al., 2012). During the past decades, improvement in surgical skills and oncological treatment has influenced the impact on survival (Kapiteijn et al., 2002; Rougier. Et al., 2003). For patients with the disease at medium or late stages, overall survival is significantly improved with adjuvant therapy after surgical resection. In particular, fluorouracil-based therapies (5-fluorouracil/leucovorin or capecitabine) have shown survival benefits to medium stage CRCs with moderate risk, and even more to later stages of the disease when associated to oxaliplatin (André et al., 2009; Haller et al., 2011). In several clinical trials, pre-operative chemotherapy, also called neoadjuvant therapy, has been reported to downstage cancer, allowing its surgical resection. In fact, a study from Pozzo et al. reported

that a combination of 5- fluorouracil/leucovorin and irinotecan allowed about 30% of the patients to have a resectable tumour, which increased the survival of 19 months.

In early-stage tumors (UICC stage I), radical hemicolectomy with lymph node resection without any additional treatment is appropriate, especially the MSIH subset (Ribic et al., 2003; André et al.,2009)

In stage II patients the gain in 5-year survival rate (2% to 3%) by adjuvant chemotherapy 5-FU or capecitabine is small (Gray et al., 2007). Tumors with a defective mismatch repair do not appear to benefit from a 5-FU adjuvant monotherapy and have excellent prognosis and are not recommended for adjuvant therapy (Sargent et al., 2010)

Patients with stage III, with lymph node metastasis adjuvant chemotherapy is administered in an attempt to eliminate microscopic metastases and thereby reduce the risk of recurrence. 5-FU-based treatment give an increase in 5 year survival rates of about 10% to 15%. In different cases the addition of oxaliplatin to a capecitabine regimen added another 4% of 3-year DFS to a bolus 5-FU-based regimen increased benefit in patients (Haller et al,. 2011). For patients older than 70 years 5 –FU-based therapy has shown benefit (Sargent el al., 2001).

Patients with UICC stage IV prognosis have poor outcomes, the 5-years survival rates are only 6%. Treatment with %-FU and leucovorin increased up to 12 months a median overall survival in metastatic, unresectable patients (Petrelli et al., 1989). The addition of irinotecan and oxaliplatin raised overall survival to a median of about 20 months (Tournigand et al., 2004) but with addition of biologicals, such as VEGF-A antibody bevacizumab or the epidermal growth factor receptor (EGFR) antibodies cetuximab and panitumumab, can arise up to 24 months (van Cutsem E et al., 2011; Saltz et al., 2008; Douillard et al 2014).

1.1.5.1 TARGETING ONCOGENIC SIGNALLING PATHWAYS

Targeted therapy includes a novel group of treatments that may help improve outcomes for patients with metastatic colon cancer as monotherapy and, in some cases, when used in combination with chemotherapy.

Promising results have been reported by new chemotherapeutic regiments and novel targeting molecules, mostly in the past few years, but 5-year survival of patients with advanced or metastatic disease, that account for 19% of all CRC at the time of diagnosis, is only the 11% (Siegel et al., 2012; Howlander et al., 2014). Novel and innovative target therapy could improve the outcome of CRC, especially in later stages, similarly to the impact that targeting agents has had on breast cancer.

1.1.5.1.1 EGFR

This receptor is overexpressed in 65-70% of all CRCs and its status is associated with tumour's advanced stages.

EGFR signalling can be blocked by preventing its activation from external stimuli or by inhibiting its kinase activity. Cetuximab, a IgG1 recombinant chimeric monoclonal antibody, and panitumumab, a IgG2 recombinant human monoclonal antibody, are the two FDA-approved antagonist antibodies targeting EGFR used in the CRC clinical practice (Cancer, 2016). They act by binding the extracellular part of the receptor, inducing its internalisation and degradation, thus inhibiting its activation and downstream signalling.

Patients in which conventional chemotherapy fails, the anti –epidermal growth factor receptor (EGFR) monoclonal antibody (moAbs) panitumumab prolongs survival (Van Cutsem et al., 2007). Approximately 10% patient achieve benefits with anti- EGFR moAbs (Cunningham et al., 2004). Different studies have reported that, in mCRC patient, tumor response to the EGFR

targeted-moAbs cetuximab and panitumumab increase in tumors where there is an increased gene copy number (GCN) of the EGFR (Moroni et al., 2005; Liévre et al., 2006; Lenz et al., 2006). Therefore, Sartore-Bianchi and colleagues, showed that the benefit of adding cetuximab to a chemotherapy is functional in patients where KRAS exon 2 have not mutation (Sartore-Bianchi et al., 2007). Recently data from clinical trials prove that no benefit was achieved with cetuximab or panitumumab treatment in tumor mutant in KRAS exon 3 and 4 (Stintzing et al., 2014) taking in consideration that those additional mutations are 10% of all mCRC cases, so only the 50% of the mCRC patients are qualified for anti-EGFR treatment.

In patients with RAS wild type tumor, bevacizumab and cetuximab or panitumumab can be used.

These therapies can be used in combination such as cetuximab that associates with irinotecan and fluoropyrimidine shown a significant increase in mCRC survival (Van Cutsem et al., 2009). Instead cetuximab association with oxaliplatin have not replicated the same effect, despite a slight increase of survival (Bokemeyer et al., 2009).

Similarly, panitumumab displayed efficacy only in association with irinotecan and 5-fluorouracil (Peeters et al., 2014). Even if the efficacy of cetuximab and panitumumab has been clearly demonstrated, it remains modest, as objective response rates are comprised between 8 and 23%. The most relevant predictive markers of resistance to anti-EGFR antibodies are currently represented by somatic gene mutations, which are both implicated in colorectal carcinogenesis and responsible for a ligand-independent activation of intracellular signalling pathways downstream of EGFR.

The benefit of these therapeutic regiments was nullified in presence of KRAS mutations (Bokemeyer et al., 2009; Cutsem et al., 2010).

1.1.5.1.2 RAF

Considering that RAF mutations are usually mutually exclusive with KRAS mutations, RAF could be a suitable target for pharmacological inhibition. Clinical trials performed on metastatic melanoma demonstrated encouraging results, but response was hampered by acquired resistance to the inhibition, which reactivated MAPK activity, for example by novel mutation of MEK or NRAS (Flaherty et al., 2012). Combination of MEK and BRAF inhibitors has indeed displayed promising results in metastatic melanoma clinical trials. Moreover, targeting of EGFR and BRAF simultaneously have identified sustained suppression of MAPK pathway and enhanced anti-cancer effect in xenograft models of mutant BRAF CRCs (Corcoran et al., 2012).

1.1.5.1.3 VEGF and VEGFR

Angiogenesis is an essential physiological process that can be dysregulated in several pathological conditions, including cancer (Folkman et al., 1987; Samant et al., 2011; Ferrara et al., 2005). The vascular endothelial growth factor (VEGF) pathway is well characterized for its importance in the angiogenesis contribution.

In a trial reported by Hurwitz et al, the combination of bevacizumab with irinotecan, 5-fluorouracil (5-FU), and leucovorin (IFL) was shown to improve the survival of patients with metastatic colorectal cancer (mCRC), resulting in its approval as the first antiangiogenic therapy (Hurwitz et al., 2004; Ferrara, 2005). While 2-year survival has improved to the 24- to 28-month range, the overall prognosis of mCRC remains poor, with 5-year survival generally between 5% and 8%, despite the availability of such therapy (Chu, 2012).

Bevacizumab, the first antiangiogenesis therapy to be approved for use in mCRC, is a humanized monoclonal antibody that binds to all isoforms of VEGF. Evidence for the clinical

efficacy of bevacizumab in cancer, notably in the treatment of mCRC, has been reviewed elsewhere (Tol J, 2010). Different opinions on bevacizumab treatment can be found: while some studies have shown that the addition of bevacizumab improves survival, the results of other studies have not confirmed these findings (Allegra CJ et al., 2013). But in the last year the benefits of this treatment is well studied and it is reported that chemotherapy combined with bevacizumab significantly improved overall survival in the first-line treatment for patients with mCRC (Jang et al., 2017; Ilic et al., 2016). Therefore, important evidences about the addition efficacy of bevacizumab to the chemotherapy for patients with mCRC are suggested for the second-line treatment. By the analysis of several trials results that patients who received the combined bevacizumab and chemotherapy treatment as second-line therapy showed a longer overall survival, with no significant sever adverse event than bevacizumab-naive based chemotherapy (Ruan et al., 2019).

In the United States, bevacizumab is currently indicated in combination with intravenous 5-FU-based chemotherapy for the first- or second-line treatment of mCRC.

In pre-clinical studies, Regorafenib, a multikinase inhibitor that blocks the activity of several protein kinases, has shown antitumour activity, including in colorectal cancer models (Wilhelm et al., 2011). Understanding the clear mechanism by which patients respond positively to VEGF inhibition remains a challenge.

1.2 STARD3

STARD3 is a cholesterol-binding protein belonging to the START (steroidogenic acute regulatory protein–related lipid transfer) protein family and is involved in the shuttling of cholesterol across different membranes of the cellular compartments. In the last years several studies have demonstrated the involvement of STARD3 in cancer, specifically HER2-overexpressing breast cancer, as an oncogenic partner of HER2-driven carcinogenesis. In fact, STARD3 has been discovered during a screening of lymph nodes derived from metastatic breast cancer, hence the name MLN64 (metastatic lymph node 64). STARD3 gene is located in the q11-q21 of chromosome 17. This region is altered in almost 20-30% of breast cancers and the most common amplification is about the proto-oncogene erb 2 (HER2).

STARD3 is a member of a subfamily of lipid trafficking proteins characterized by a C-terminal steroidogenic acute regulatory domain (STARD), which shares a 35% of homology with the domain of StAR protein, STARD1, a transporter of cholesterol in mitochondria. They both belong to the START (steroidogenic acute regulatory protein–related lipid transfer) proteins family, involved in the non-vesicular transport of lipids in membranes (Alpy et al., 2005).

STARD3 is formed by two major domains, MENTAL, which has a FFAT motif, and START. On the C-terminal side, it is localized the START domain, by which it exerts steroidogenic activity. The steroidogenic event exerted by START domain seems to take place in the mitochondria. It has been reported that mitochondrial proteases could process STARD3 producing a 28 KDa fragment corresponding to the START domain, which stimulates the synthesis of steroids, such as progesterone (Esparza-Perusquía et al., 2015). The crystal structure of the START domain of STARD3 revealed a hydrophobic cavity formed by the α/β helix grip structure of the 210 amino acids which binds one molecule of cholesterol at an equimolar ratio 1:1 (Tsujishita Y, 2000), transporting sterol from the endoplasmic reticulum

(ER) to the endosomes (Clark BJ, 2012; Wilhelm LP et al., 2017). Several roles of START have already been discussed extensively in the next section.

MENTAL domain is composed of four transmembrane helices and is located at the N-terminal of the protein, it distinguishes STARD3 from the other START domain proteins (Alpy 2002). It is present also only in STARD3 N-terminal like (STRAD3NL), with whom it shares more than 80% of homology. It prompts the localization of STARD3, by the interaction with 14-3-3 protein, in late endosome (LE), with a particular accumulation in the limiting membrane (Liapis et al., 2012). Whereas no proper signal for this localization has been identified, a mutated version of MENTAL showed the inability of STARD3 to bind to the LE. MENTAL domain seems to have the cholesterol binding activity as well as the START domain, promoting the formation of cholesterol micro-domain between the helices, supporting the notion that is a sterol reservoir (Alpy et al., 2005). The MENTAL domain is probably involved in the association between LE and ER. In fact, the domain contains a conserved diphenylamine (FF) in an acidic tract (FFAT)-like motif. By FFAT motifs, STARD3 interact with vesicle associated membrane protein (VAMP) (Alpy 2013).

STARD3 is localized in the outer periphery of LE directly facing the ER, creating specific subcellular regions named membrane contact sites (MCSs) (Alpy et al., 2013). Because of its localization in the surface of the ER, STARD3 was initially proposed to facilitate cholesterol exit from this organelle. New evidences clarify that STARD3 transports sterol from the ER to the endosome by its ability to bind sterol and to create MCSs. This cholesterol redistribution is at the expense of the plasmatic membrane favoring membrane formation inside endosomes. In this way they described a new pathway for sterol fluxes (Wilhelm 2017). Conversely, STARD3 is believed to transferring cholesterol from LE to ER across the MCS (Figure 6).

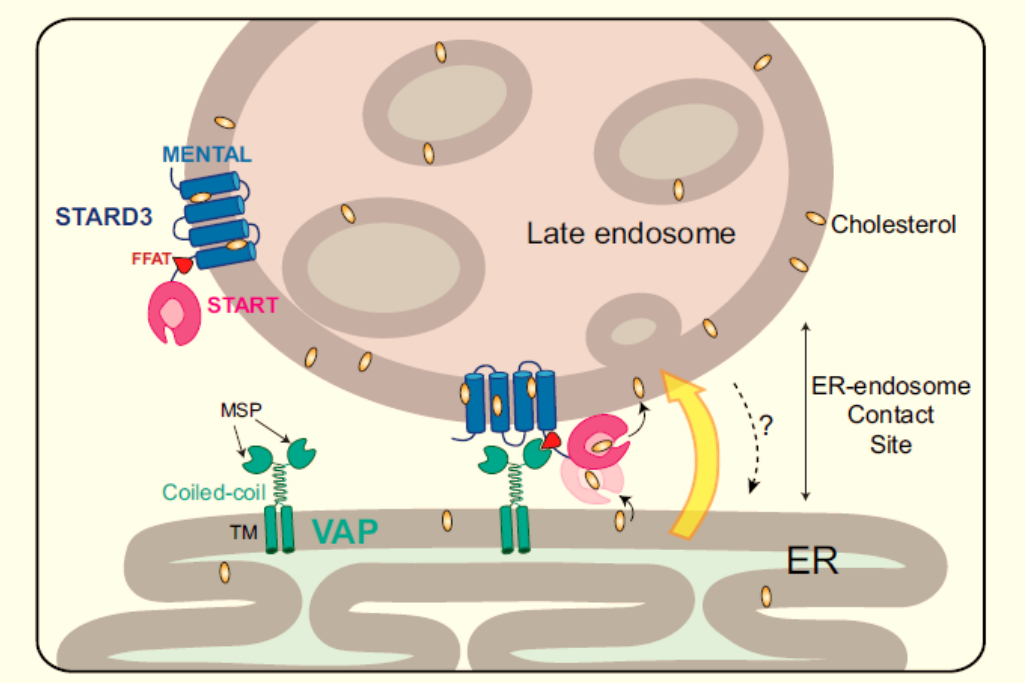


Figure 6. Mechanism of action of STARD3. STARD3 acts on intracellular cholesterol repartition by mediating cholesterol transport from the endoplasmic reticulum (ER) to endosomes at the expense of the plasmatic membrane (PM). STARD3 action relies both on its ability to create contact sites between the ER and endosomes via the direct interaction with VAP proteins, and on the sterol exchange capacity of the START domain. Adapted from Wilhelm et al., 2017

1.2.1 THE START DOMAIN PROTEIN FAMILY

It has long been appreciated that maintaining proper cholesterol distribution within the cell is important for cholesterol homeostasis and membrane function (Qin et al. 2006; Maxfield and van Meer, 2010). There are two major gene families for lipid transfer proteins with specificity for sterols: the steroidogenic acute regulatory protein (StAR)-related lipid-transfer (START) domain family and the oxysterol-binding protein (OSBP) family, which includes the OSBP-related proteins (ORPs).

The mammalian START domain protein family is well characterized and is composed of 15 members that group into six subfamilies based on the sequence and ligand similarities (Ponting - Aravind 1999; Soccio et al. 2002).

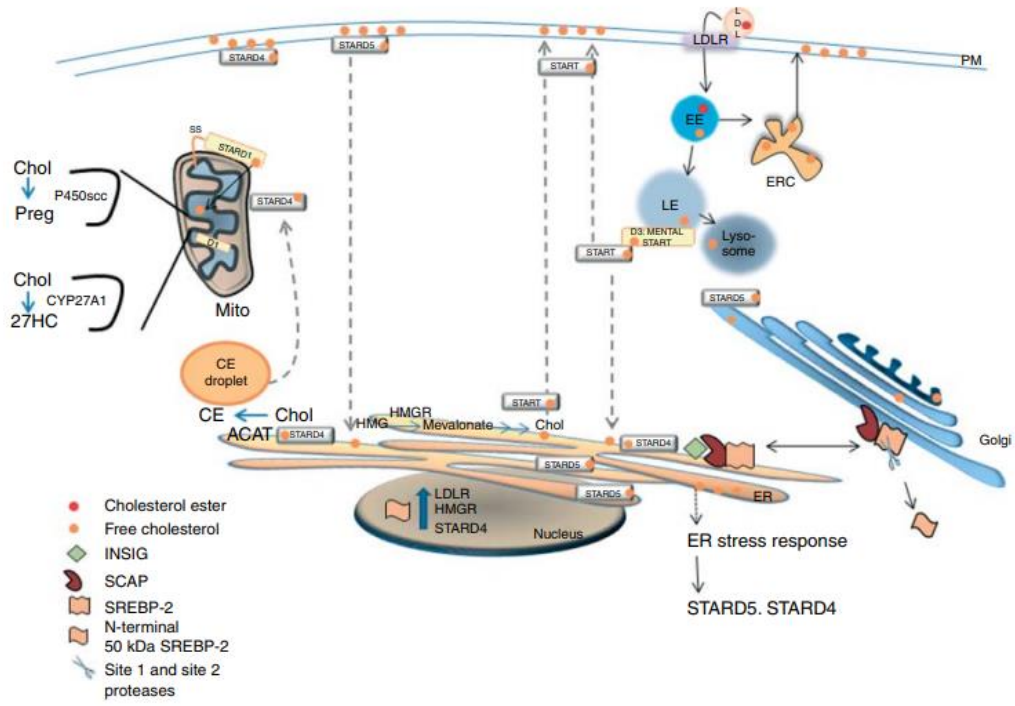


Figure 7. Model for non-vesicular cholesterol trafficking by the START domain proteins. The cholesterol-binding START proteins are shown at subcellular locations identified by immunohistochemistry. Adapted from Clark et al., 2012

The START domain is defined by a conserved sequence of ~210 amino acids that folds into an α/β helix-grip structure forming a hydrophobic pocket for binding sterols and other lipids (Iyer et al. 2001).

In very general terms, the subfamilies can be classified into cholesterol- and oxysterol binding proteins (STARD1/D3 and STARD4/D5/D6 subfamilies), the phospholipid- and sphingolipid-binding proteins (STARD2 (phosphatidylcholine transfer protein, PCTP)/D7/D10/D11 subfamily), the multi-domain proteins containing either putative Rho-GTPase signaling function (STARD8/12/13 subfamily) or thioesterase activity (STARD14/15 subfamily), and the STARD9 subfamily composed of a single member of unknown function that is not further discussed (Soccio et al., 2002; Soccio and Breslow 2003; Alpy and Tomasetto, 2005) (Figure 7). The crystal structures for the START domains of STARD3 protein) and mSTARD4 were the first to be solved and showed an α/β helix-grip fold with a nine-stranded anti-parallel β-sheet forming a U-shaped hydrophobic cleft that binds the ligand and is flanked by amino- and carboxyl-terminal α helices (Tsujishita & Hurley 2000, Romanowski et al. 2002). The carboxyl-terminal α helix is proposed to serve as a 'cap' to the ligand-binding site, with lipid access to the binding pocket requiring a conformational change in the START domain and movement of the C-terminal helix (Baker et al. 2005; Bose et al. 2008).

StAR is the founding member of the START domain protein family. Together with STARD3 they are the first subfamily. The two member are similar in the START domain and both proteins binds only cholesterol. The differential localization in the subcellular compartments suggests different function in cholesterol trafficking. StAR/STARD1, and is expressed predominantly in the adrenal and gonads where it functions to bind cholesterol and facilitate its transfer from the outer to the inner mitochondrial membrane to initiate steroid hormone biosynthesis. Mutations STARD1 gene are the most common responsible for congenital lipoid

adrenal hyperplasia (lipoid CAH; King et al., 2011). This disorder is characterized by the inability to synthesize adrenal or gonadal steroid hormones due to the absence of cholesterol transport into mitochondria. STARD1 knockout mice confirmed that in the absence of the protein the animals die shortly after birth due to the absence of adrenal hormones (Caron et al., 1997). Re-expression of STARD1 transgene in the knockout mice fully restore adrenal and gonadal steroidogenesis. Mice that expressed an amino terminal truncated STARD1 that was not targeted to the mitochondria had partially restored steroidogenesis in a tissue and gender specific manner and retained lipid accumulation in the adrenal and gonads (Sasaki et al., 2008). Several was previously discussed about STARD3. In addition, homozygous STARD3 mutant mice that express a STARD3 protein containing the N-terminal MENTAL domain but lacking the START domain do not accumulate cholesterol in late endosomes/lysosomes and synthesize steroid hormones at wild-type levels (Kishida et al. 2004). Other proteins, implicated in the sterol transport such as Stard4, Stard5, Npc1, and Npc2 mRNA levels were not changed due to loss of the START domain from STARD3, suggesting that the lack of a phenotype was not due to compensatory increases of these cholesterol transporters. However, whether the intact MENTAL domain of STARD3 may be responsible for the function of this transporter in late endosomes in the knockout mice, or whether STARD4 or STARD5 can act as the soluble cytoplasmic acceptor of cholesterol from STARD3/MLN64 or NPC1, remains to be determined.

The other group is the STARD4 subfamily, composed of STARD4, STARD5, and STARD6. This subgroup share ~20% sequence identity with STARD1/D3 subfamily (Soccio et al. 2002). STARD4 was identified as a novel EST in a cDNA microarray study designed to identify cholesterol-regulated genes in mouse liver. STARD5 and STARD6 were identified from a BLAST search of the human genome against STARD4. STARD4 is the main component of the subfamily, it plays a role in the shuttling of sterols to endoplasmic reticulum

(ER) (Alpy and Tomasetto,2005; Rodriguez-Agudo et al., 2011). In fact, the cellular cholesterol levels are strictly linked to ER cholesterol and an increase induces esterification of cholesterol, whereas a decrease lead to the activation of sterol regulatory element-binding protein-2 (SREBP2) (Goldstein et al., 2006). When there is an increase in cholesterol levels, the transcription factor precursor SREBP2, is inactive and sequestered in ER, but when the sterol levels decrease, a pathway of the cholesterol uptake is activated and genes involved in cholesterol synthesis, and, by a mechanism of negative feedback, STARD4 itself. This is further demonstrated by STARD4 overexpression, which results in SREBP2 retention in ER (Goldstein et al., 2006; Rodriguez-Agudo et al., 2011). Mice fed a high-cholesterol diet had reduced Stard4 transcript levels with Stard4 gene expression later shown to be regulated by a SREBP2-dependent mechanism (Soccio et al. 2002, 2005, Rodriguez-Agudo et al. 2011). Therefore, STARD4 overexpression in primary mouse hepatocytes increased bile acid synthesis and cholesterol ester synthesis (Rodriguez-Agudo et al. 2008), indicating increased cholesterol transport to mitochondria and ER. Homozygous STARD4 knockout mice do not present with a strong lipid phenotype; the plasma and hepatic lipid content for both male and female STARD4 null mice is comparable to its wild-type counterparts (Riegelhaupt et al. 2010). STARD5 binds with greater affinity to cholic and chenodeoxycholic acid, components of bile acids, than cholesterol and hydroxycholesterol (Létourneau et al., 2012; Mesmin et al., 2013). Nevertheless, evidences suggest that its role may be transporting cholesterol to mitochondria and ER, increasing free cholesterol levels and inducing the transcription of SREBP2 (Borthwick et al., 2010; Rodriguez-Agudo et al., 2008). Overexpression of human STARD5 in primary rat hepatocytes resulted in increased cellular-free cholesterol levels with possible increased ER cholesterol content (Rodriguez-Agudo et al. 2005). Instead little is known about STARD6.

The phospholipid/ceramide subgroup presents structural and ligand-affinity differences between the components. STARD2/PCTP, STARD7 and STARD10, STARD-only proteins, bind to phosphatidylcholine (PC), instead STARD11/CERT, which present two membrane-interacting domains, binds to ceramides. All these proteins are expressed ubiquitously (Hanada et al., 2003; Horibata and Sugimoto, 2010; Kanno et al., 2007; Olayioye et al., 2005). STARD2, or PC-TP, is a protein that binds and rapidly transport PC to the plasma membrane and the mitochondria (Kang et al., 2010). It is usually localized in cytoplasm, but it can be also detected in mitochondria. The second component of the subgroup is STARD7 which is involved in PC shuttling to the mitochondria (Flores-Martin et al., 2013). STARD10 has affinity not only for PC but also for phosphatidylethanolamine (PE). STARD11, also known as CERT. Current data propose that this protein acts as a ceramide carrier from ER to Golgi, where in turn ceramide is converted into sphingomyelin or ceramide derivatives (Hanada et al., 2003). The peculiarity of STARD11 compared to the other member of the subgroup is the multi-domain structure.

The subfamily of RhoGAP-START, referred also as deleted in liver cancer (DLC), is composed by STARD12/DLC-1, STAD13/DLC-2, and STARD8/DLC-3. These proteins act as tumour suppressors in different types of cancer and share a unique multidomain structure, which consists of an N-terminal sterile alpha motif (SAM), a RhoGAP domain and a C-terminal START domain (Lukasik et al., 2011). STARD12/DLC-1 was first isolated as a genomic clone that was localized on chromosome 8p21.3–22, a region associated with loss of heterozygosity in several cancers and shown to be deleted in 50% of primary human hepatocellular carcinoma tumor tissues (Yuan et al. 1998). Re-expression of DLC-1 in human liver, lung, breast, and ovarian cancer cell lines suppresses cell growth and increases apoptosis, supporting DLC-1 as a tumor suppressor. Targeted deletion of Stard12/dlc-1 gene

in mice results in embryonic lethality, most likely due to disruption of cytoskeletal organization.

STARD13/DLC-2 and STARD8/DLC-3 also have tumor suppressor activities when overexpressed in cancer cell lines (Ching et al. 2003, Durkin et al. 2007a,b) and localize to focal adhesions (Kawai et al. 2007, 2009). Recent reports characterizing STARD13/DLC-2 knockout mice show that the mice are healthy and fertile with no overt phenotype (Yau et al. 2009, Lin et al. 2010). The knockout mice were not more susceptible to spontaneous tumors or induced hepatocarcinogenesis, indicating potential compensatory effects of the other DLCs for tumor suppressor activity or possible requirement for a 'second hit' to promote tumor formation (Yau et al. 2009, Lin et al. 2010). The last group is represented by thioesterase subfamily, which is composed by two proteins: STARD14 and 15. They belong to the acyl-CoA thioesterase from which they differ only for the presence of the START domain and possess two hotdog-fold/thioesterase domains (Kirkby et al., 2010). STARD14 acts as a dimer, favouring the binding to long-chain fatty acyl-CoAs, localizes in cytosol and microsomes (Han and Cohen, 2012). Finally, the last member of the START-domain containing protein is STARD9, its role is not fully understood, it localises to the centrosomes and seems to be important for mitosis, since STARD9 knock-down induces aberrant mitosis and, in turn, apoptosis. Moreover, its silencing promoted apoptosis after taxol treatment, therefore suggesting a possible target of therapeutic intervention (Torres et al., 2011).

1.2.3 STARD3 AND CANCER

Although the functions and structures of the components of this family is not totally clarify and discovered, it is well reported the importance of several components in cancer: in this regard STARD1 plays a role in the chemotherapy resistance of the hepatocellular carcinoma (Montero J. et al., 2008) and mutations in this gene are the common basis for the onset of congenital lipoid adrenal hyperplasia (CLAH), caused by the absence of cholesterol transport into mitochondria (King SR. et al., 2011); on the other side, the deletion of the region associated with STARD12, a START protein with a multi domain, were found in 50% of the primary hepatocellular carcinoma tissue (Yuan BZ. Et al., 1998), and the re-expression of the protein in human liver, lung, breast, and ovarian cancer cell lines suppresses cell growth and increases apoptosis, supporting STARD12 as a tumor suppressor.

In human, it was demonstrated that STARD3 is overexpressed in different cancer cell lines and in particular, with Her2 overexpressing breast cancer (Alpy and Tomasetto, 2014). In fact, STARD3 and HER2 are co-amplified and co-overexpressed in about 25% of breast cancers (Dressman et al., 2003). The molecular mechanism by which STARD3 cooperate with HER2 is still unclear (Sahlberg KK. Et al., 2013).

Nevertheless, this gene is implicated in therapy resistance of breast cancer and clinical results of Her2 positive breast cancer patients with poor outcomes, metastasis , local recurrence and shorter overall survival showed high level of STAR3 trasncript (Vinatzer U, et al., 2005; Lamy PJ. Et al., 2011; Cai W., et al., 2010). This has been supported by STARD3 silencing in HER2 positive breast cancer cell lines with a reduction of cell viability and increased cell death (Kao J. et al., 2006), reinforcing the idea that STARD3 can be implicated in the intratumoral steroidogenesis and a cancer progression (Watari H. et al 1997)

Recently, new evidences suggested a possible STARD3 overexpression also in colorectal, prostate and gastric cancers (Cancer Genome Atlas Network, 2012; Stigliano A. et al., 2007;

Qiu Y. et al., 2014). Elevated level of STARD3 was found in tubular and papillary adenocarcinoma cells supposing that STARD3 affecting cholesterol metabolism in gastric cancer tissues by means of increasing cholesterol transport to mitochondria and consequently activating steroidogenesis (Qiu Y. et al., 2014). In neoplastic prostate tissue, STARD3 is co expressed with another gene involved in an androgen synthesis, CYT7. Even if the link is not clear, STARD3 expression seems to be correlated with high stage, high Gleason score and short relapse-free time in prostate cancer patients.

1.3 VIRTUAL SCREENING

In virtual screening, large libraries of drug-like compounds that are commercially available are computationally screened against targets of known structure, and those that are predicted to bind with high affinity are experimentally tested (Lavecchia A et al., 2013; Reddy et al., 2007). To be selected as candidate active compound for the protein target, a popular approach to virtual screening is based on searching molecules having shapes similar to the structures of one or multiple ligands that bind to a target protein pocket .

1.3.1 VS1

The start point of this work is the crystal structure of STARD3-START domain obtained by Tsujishita, that revealed the presence of a wide hydrophobic pocket in which only one molecule of cholesterol is able to accommodate. Furthermore, by performing titration experiments they proved that START domain bind cholesterol at 1:08 ratio. No clear data about the binding disposition of cholesterol into the binding site are reported in literature until now. By a collaboration with the University of Pisa, a consensus docking procedure was employed for predict the reliable binding mode for cholesterol into the binding site. These computational protocols consist in docking ligands using multiple docking methods and the binding poses predicted were compared and clustered together to search for common binding modes: the docking poses that shows the highest consensus level were chosen as the final predicted binding mode. The docked poses obtained were investigate through molecular dynamics simulations and a detailed analysis revealed that in a specific pose the cholesterol moved from its starting position allowing the formation of an H-bond between the hydroxyl group of the molecule and residue S362. As well described in literature (Murcia et al., 2006) this specific H-bond appeared to be important for cholesterol binding to the STARD3 domain, so the obtained binding mode seemed to be in agreement with the Murcia's proposed. A

virtual screening was than developed taking in consideration the results obtained and a pharmacore model was used to filter the Enamine database. Only four potential ligand were purchased after all the filters applied and tested for STARD3 inhibitory activity through a competition binding fluorescent assay. Among all compounds, VS1 was chosen after the IC_{50} values obtained, the only one able to inhibit the interaction of cholesterol with a value lower than 100 μM. For this study, a computational protocol including consensus docking, molecular dynamic simulations and binding free energy evaluations was employed to predict a reliable binding mode of cholesterol into STARD3. The results obtained allowed the development of a receptor-based pharmacophore screening that led to the identification of a lead compound (VS1) endowed with an interesting activity and thus representing the first reported STARD3 inhibitor.

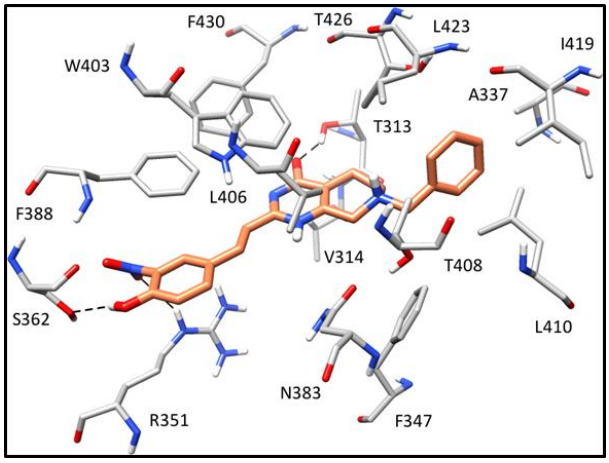


Figure 8 Minimized average structure of compound VS1 bound to STARD3 binding site. Data from Tuccinardi's lab, University of Pisa.

Structurally, this compound presents a parahydroxyl group that forms a stable hydrogen bond with the key residue S362 and in the center a bicyclic portion that interacts with the residues on the cavity. It's interesting the phenyl ring, which mimic the terminal aliphatic chain of cholesterol (Figure 8). The activity and characterization of the compound will be next presented.

2. RESULTS AND DISCUSSION

2.1 RATIONALE

To identify novel therapeutic targets, genes involved in different stages of cancer progression such as invasion, angiogenesis, and metastasis have to be characterized. Investigations of such targets, in the past, have been applied with limited success (Hamilton, S.R. et al., 2000). An emerging challenge is the identification of new genes involved in cancer development and progression in order to develop new therapeutic molecules to be used alone or in combination with current therapies. In the last years, some research groups have focused their attention on a protein initially found to be overexpressed in breast cancer: the StAR related lipid transfer domain containing 3 (STARD3). Due to its involvement in cancer, STARD3 is an attractive candidate as a target for cancer therapy and the identification of selective STARD3 inhibitors is an interesting but yet undiscovered field of study.

Keeping in mind the power of Virtual Screening to find new pharmacological drugs, we thought to identify, in collaboration with the University of Pisa (Prof. Tiziano Tuccinardi), a first STARD3 inhibitor: VS1. In this study, starting from preliminary data in which STARD3 knockdown in colorectal and breast cancer cells arise in important reduction of cell viability, we have characterized this inhibitor from its specificity for START domain to its activity in both cancer cells.

2.2 STARD3 affects cell viability of colon and breast cancer cell lines.

In order to test the effect of STARD3 silencing, we infected colon (HCT-116) and breast cancer cell line (MDA-MB-231) with three different lentiviral vectors: two were able to down regulate STARD3 and one was the control empty vector (PLKO). First of all, we evaluated the variation of cell viability. The effect of knockdown on the viability was monitored every 24 hours, for 96 hours and from the analysis, we observed a substantial and robust reduction of vitality in the entire cells knockdown for STARD3 compared to the control (Figure 9). By the analysis, it was evident that STARD3 downregulation had a fundamental effect on cell proliferation. In fact, its ablation caused an important decrease of vitality in the cell lines. Although previously observed in breast cancer, we identified the effect of STARD3 on viability of colorectal cancer cells, and given its profound consequences when silenced, we speculated on its possible involvement in carcinogenesis as an oncogene.

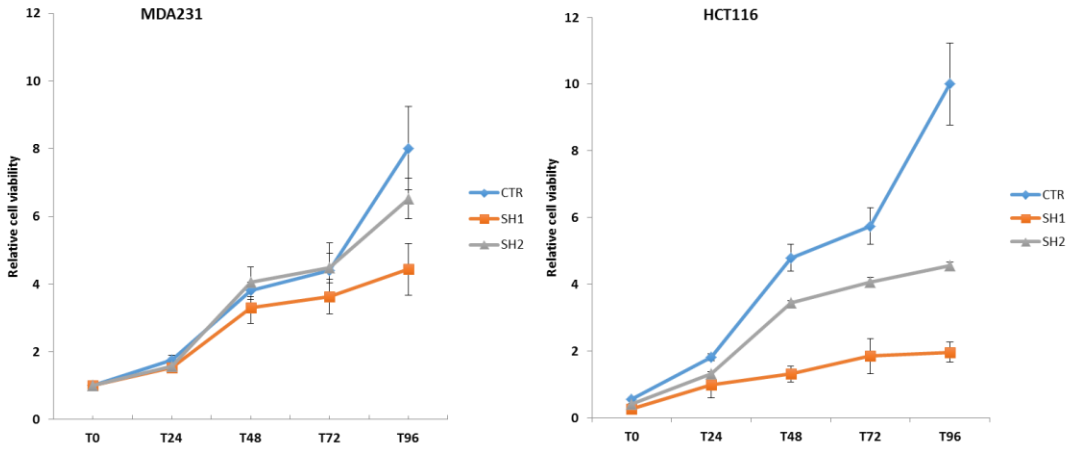


Figure 9 STARD3 silencing hampers cell viability. Relative viability of HCT116 and MDA-MB-231 transduced with two different shRNAs compared to the control. The cells were seeded at 500 cells/well (HCT116) and 1000 cells/well (MDA-MB-231) and the vitality was measured by luminescence over a 96-hours period, every 24 hours, using a microplate reader. The relative cell viability was normalised on the values of cells transduced with empty vector (CTR).

By western blot analysis, we monitored the decrease of the level of the protein STARD3, indicating the specificity of the shRNA activity and it was evident the downregulation of the protein (Figure 10).

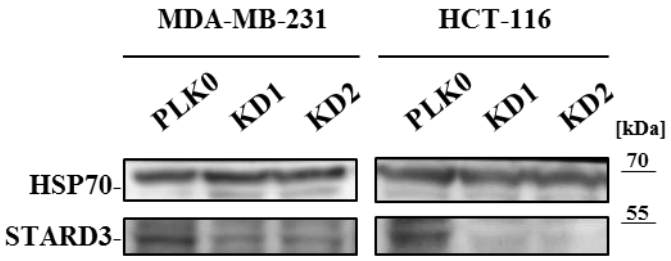


Figure 10. Western blot analysis to validate the STARD3 expression in different cell lines.

2.3 HIS-START PURIFICATION AND BIOCHEMICAL ASSAY

Taking in consideration the binding of cholesterol to STARD3 with a stoichiometry of 0.8:1 and the concentration of protein and substrate utilized in the assay developed by Tsujishita Y. and Hurley J.H (Hurley & Tsujishita, 2000) a competition binding fluorescent assay was performed. In order to proceed with the assay, a plasmid contain START domain of STARD3 protein was expressed into BL21 cells and purified with HIS-tag beads. The HIS-START protein was eluted with increasing concentration of imidazole (from 10 to 400 mM). Equal amount of samples (5 μl) were loaded into 12% SDS PAGE in order to check the purity and BSA was used as control of concentration. As shown in figure 11, we obtained the best affinity and elution with the 100-200 mM of imidazole.

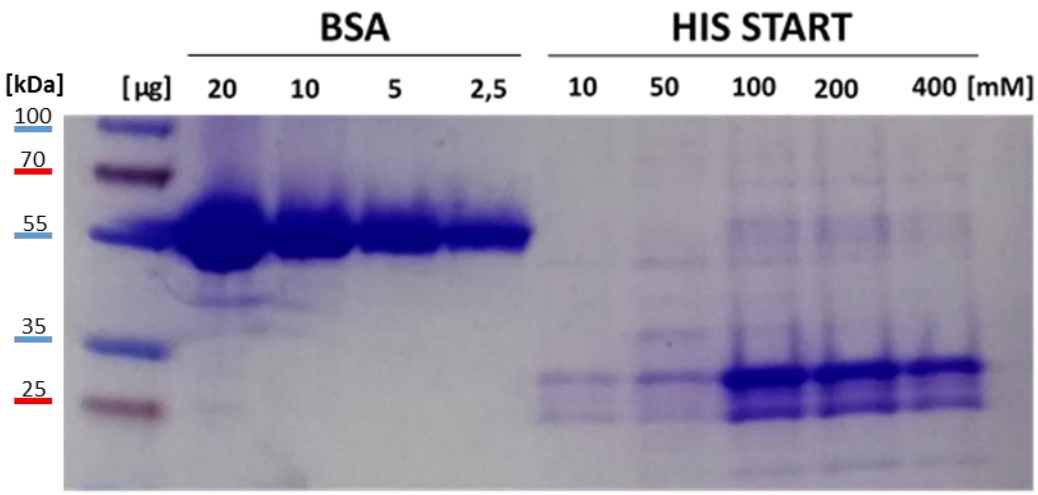


Figure 11. SDS PAGE of HIS START. Five elutions were obtained with different Imidazole concentration (10, 50, 100, 200, 400 mM). Equal amounts of samples were loaded into 12% gel to check the purity of the samples. BSA was used as control of the concentration.

The competition binding fluorescent assay was performed using as a substrate 3-hexanoyl-NBD Cholesterol. The value of IC50 were reported in Table 1. From the analysis was evident the high specificity for the active domain of the protein. With an IC50 of 35 uM, the inhibitor is able to bind the domain and to inhibit the interaction with the cholesterol.

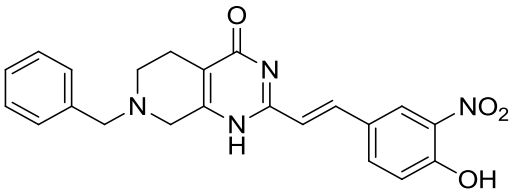
	structure	**IC50 Av ± Sdev**
VS1		35±4.7

Table 1. Structure and activity of the selected compound

2.4 Compound VS1 affects cancer cell viability

To examine the potential therapeutic effects of STARD3 inhibition, VS1 was then tested in cancer cells. Breast (MCF7 and MDA-MB-231) and colon (HCT-116) cancer cell lines were selected as models. (Vassilev B. et al., 2015; Cai W., et al., 2010; Uhlen M. et al., 2013)

Cell line	Activity (μM) Av±sDev
MCF7	**105.5 ± 10.4**
HCT116	**70.7 ± 4.5**
MDA-MB-231	**49.7 ± 6.8**

Table 2. Anti-proliferative activity of VS1 on representative breast and colon cancer cell lines

As reported in Table 2, VS1 showed an interesting activity inhibiting cell viability of all cell lines in the low micromolar range.

To determine the effectiveness of cytotoxic agents, the survival of a single cancer cell could be tested through a clonogenic assay. For this purpose, MCF7, MDA-MB-231 and HCT-116 cell lines were treated with different concentrations of VS1 and grown until colonies were visible. Results shown in Figure 12B demonstrated that VS1 could impair the growth of cancer cells in a dose dependent manner. To confirm the specificity of this result, STARD3 was knocked down with two shRNAs. Both shRNAs inhibited the growth of cancer cells, confirming that VS1 is a valid inhibitor of STARD3 (Figure 12A).

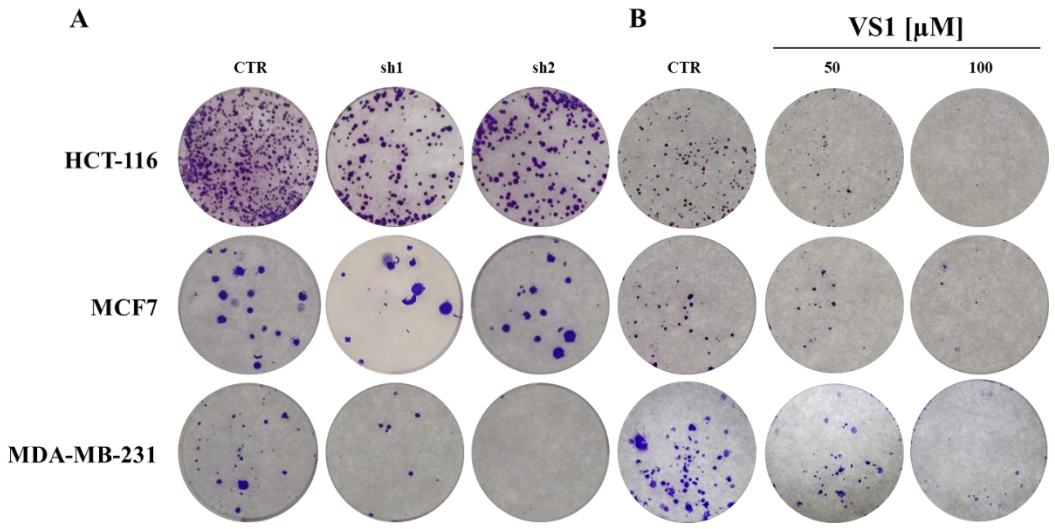


Figure 12. Colony assay in different cell lines. A) Cells were treated with two specific shRNAs for STARD3 or B) with different doses of VS1. Colony forming efficiency was evaluated by visual inspection.

2.5 Inhibition of STARD3 induces Caspase-3 and -7 activities

Apoptosis levels were investigated in HCT-116 and MDA-MB-231 upon STARD3 silencing by VS1 treatment.

VS1 treated cells with the maximum dose were collected 24 and 48 hours after treatmentn. The protein lysates were incubated with Caspase-Glo 3/7® kit reagents and luminescence measured by microplate reader using a luminescence assay that detected caspase activity.

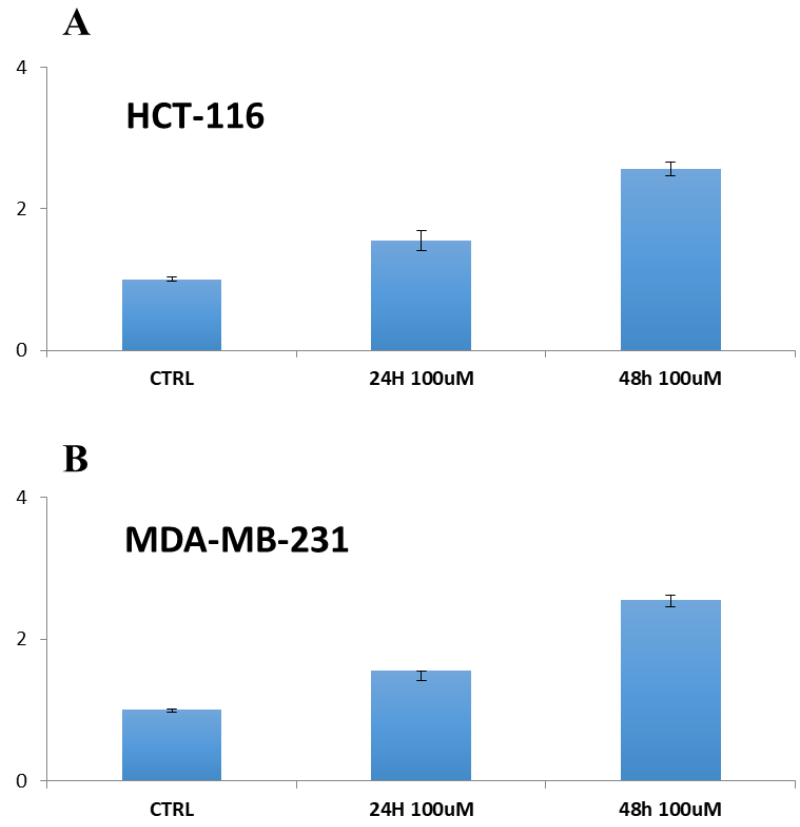


Figure 13. STARD3 prevents apoptosis by inhibiting Caspase-3 and -7 activity. Relative caspase activity of HCT116 (a), and MDA-MB-231 (b) treated with VS1 at 24 and 48 hours and control cells, analysed by luminescence assay.

Both HCT-116 cells and MDA-MB-231 cells, STARD3 inhibition shows an increasing of caspase 3-7 in particular after 48h after VS1 treatment (Figure 13A/B). We showed a clear involvement of STARD3 in the apoptotic process in colorectal cancer, which suggest that STARD3 may play a role as anti-apoptotic protein, another hallmark of cancer, thus supporting the oncogenic potential of this gene.

2.6 Compound VS1 induces STARD3 protein degradation

It has been reported that high affinity or covalent inhibitors induce degradation of target protein (Long el al., 2012; Russo Spena, et al., 2018). Since NIH3T3 cell line well tolerated protein synthesis inhibitor cycloheximide (CHX), VS1 was used in combination with CHX. Compared to control, VS1 reduced the level of STARD3 protein in CHX treated cells (Figure 14A). Moreover, the mRNA level of STARD3 was unaffected by the treatment (Figure 14B).

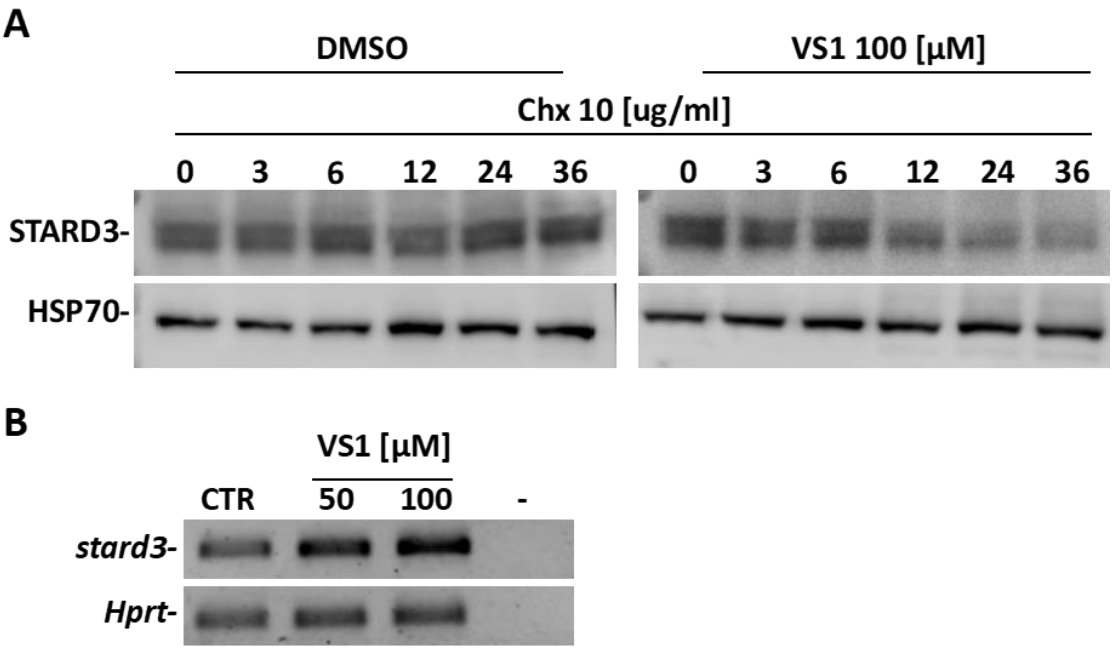


Figure 14 Compound VS1 decrease STARD3 protein stability. A) NIH3T3 fibroblasts were treated with 100 µM of compound VS1 for 24 h followed by 10 µg/mL of CHX for the indicated time (hours). B) Cells were treated as in (A) at two different concentration of VS1. STARD3 RNA levels was unaffected.

2.7 Stard3 inhibition increased the level of FAK

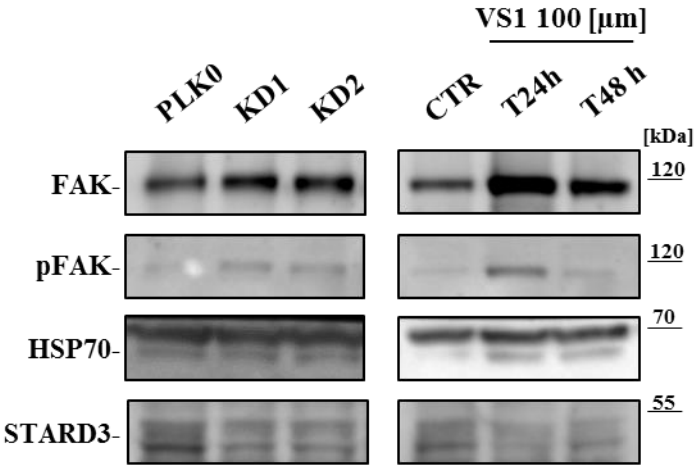


Figure 15. MDA-MB-231 cell line was treated with 100 µM of compound VS1 for 24 and 48 hours. Western blot analysis shows that the level of fak and pTyr397-FAK was increased in treated cells. Among STARD3 target proteins, Focal adhesion kinase (FAK) was demonstrated to be regulated in MDA-MB-231 cells. Here we confirm that inhibition of STARD3 increased the level of FAK and pFAK

FAK is a non-receptor tyrosine kinase, which plays a key role at focal adhesion sites by promoting cell spreading, migration, and the transmission of anchorage-dependent anti apoptotic signals (McLean et al., 2005). Its auto-phosphorylation at the Tyr-397 site is an important event for maintaining the biological function of FAK, because it creates a high-affinity binding site for proteins with SH2 domains, including the Src family kinases, which will further phosphorylate FAK on other tyrosine residues, such as Tyr-576 and 577, to positively up-regulate FAK activity (Van Nimwegen et al., 2007). FAK is an important mediator of cell proliferation, migration, and survival, and any perturbation of these processes is often associated with the development of malignancy.

In fact, increased FAK levels have been reported in many types of cancers, including prostate, cervix, colon, ovary, and breast cancer (McLean et al., 2005).

Finally, since FAK was demonstrated to be regulated by STARD3 in MDA-MB-231 cells (Cai et al., 2010), we evaluated the levels of FAK and pTyr397-FAK in VS1-treated MDA-MB-231 cells. Cells were treated with 100 μM of compound VS1 for 24 and 48 hours. The lysates were processed for western blot analysis and the expression of FAK and pTyr397-FAK was evaluated. The results confirmed that inhibition of STARD3 by VS1 increased the level of FAK and pTyr397-FAK (Figure 15) demonstrating the important action of the compound.

3. CONCLUSIONS

Colorectal cancer remains the second leading cause of cancer death in the United States. Survival for patients with metastatic colorectal cancer, however, has improved dramatically over the past decade. But the necessity to better therapies get researchers agree, remaining one of the major challenges.

On these bases, we wanted to investigate the role of STARD3 as a possible oncogene involved in colorectal carcinogenesis. In this study, the effect of the STARD3 knockdown by drug treatment was evaluated in colorectal and breast cancers, starting from several data in which STARD3 is widely recognized as protagonist in HER2 positive breast cancer and the TCGA report of genes found to be overexpressed in CRC. For this investigation VS1 inhibitor was chosen as candidate.

The specificity of the inhibitor was evaluated by measuring the stability of STARD3 and its target protein, the focal adhesion kinase (FAK). Furthermore, the effects of VS1 were evaluated in breast and colorectal cancer cell lines, demonstrating its anticancer activity. A competition binding fluorescent assay performed for testing the inhibitory activity of the selected compounds revealed an IC50 of 35±4.7 μM for compound VS1. The biological activity of VS1 was thus evaluated by treating two breast (MCF7 and MDA- MB-231) and one colon cancer cell lines (HCT-116) with different concentrations of the inhibitor. Results showed a promising antitumoral activity of VS1 in all cell lines, with IC50 values ranging from 49.7 to 105.5 μM. The reduction of cancer cell proliferation induced by VS1 was also confirmed through clonogenic assays. Even if further investigations are needed to test the efficacy and the safety of this molecule, compound VS1 represents the first available STARD3 inhibitor endowed with a relevant biological activity in cancer cell lines. Although the IC50 of VS1 on STARD3 is in the micromolar range, the induction of STARD3 degradation and the activation of a specific STARD3 target (FAK) produced by the ligand

suggest a potent and specific activity of VS1 at cellular level. Pre-treatment of NIH3T3 fibroblast cells with VS1, followed by treatment with protein synthesis inhibitor CHX, induced the reduction of STARD3 levels over time, suggesting a potent activity of VS1 on STARD3. Moreover, in agreement with literature data, Western blot analysis confirmed the implication of STARD3 in FAK pathway, as shown by the higher levels of FAK and pTyr397-FAK in VS1- treated MDA-MB-231 cells, with respect to untreated cells.

Whereas the mechanism of STARD3 is still elusive, from our results is interesting the role of this gene in colorectal cancer cells. The inhibition of STARD3, confirmed by shRNA and drug treatment, arise in a consistent reduction of cell viability and in an induction of apoptosis, suggesting an oncogene role of STARD3 in colorectal cancer. Further investigations are needed to understand in which way STARD3 acts, studying its role in cholesterol transport and in the homeostasis of cell membranes. In fact, it's well demonstrate that alteration in cholesterol content are fundamental to the survival and growth of cancer cells as well as their ability to metastasize. For these reasons, STARD3 is an example of a new promising oncogenic target whose inhibition may provide benefits to colorectal cancer patients.

4. MATERIALS AND METHODS

shRNA PLASMID PRODUCTION

The shRNA clones were purchased in bacterial glycerol stock format. Each bacterial clone, correspondent to one shRNA, was streaked on LB agar plate, additioned with ampicillin (100 μg/ml, Sigma), and left to grow overnight at 37° C. The resulting colonies were picked, inoculated in LB medium (ThermoScientific), and cultured overnight at 37° C in an orbital incubator. The bacterial culture was centrifuged, in order to recover the pellet, at 4500 rpm for 10 min, then the supernatant was discarded. The plasmid DNA was purified from the bacterial pellet by Plasmid Midi Kit (Qiagen) following manufacturer's recommendation, and the quantity was assessed by nanodrop (Thermo Fisher Scientific) and quality with both nanodrop and on 1% agarose gel. The plasmids were stored at -20°**.**

LENTIVIRAL PRODUCTION, TRANSDUCTION AND GENERATION OF STABLE CELL LINES

To produce lentivirus, 7 × 105 HEK293T packaging cells per pool were seeded in 2 wells of 6 multiwell plate 1 day before transfection. For each well, we diluted 2 μg of Sigma-Aldrich custom library plasmid (i.e. shSTARD3), 0.5 μg of pMD2G, 1 μg of psPAX2 in 100 μl of plain DMEM incubated 20 min at RT, plus 16 μl of transfection agent (FuGENE® HD, Promega). The cells were incubated for 24 h at 37 °C, after which the medium was refreshed. Lentivirus-containing supernatants were collected at 48 and 72 hours post-transfection with 20% of FBS. The two collections of lentiviral particles were pooled, filtered through a 0.45 μm membrane (Sartorius Stedim/PVDF) and stored at −80 °C. Cell lines were transduced with lentivirus supernatants supplemented with 8 μg/ml hexabromide (Sigma). At 24 h post-infection, medium was replaced and cells were selected with 2 μg/ml of puromycin or 5 ug/ml

blasticidin (Gibco). Antibiotic selection was stopped as soon as no surviving cells remained in the no-transduction control plate.

PRIMER SELECTION

Primers were selected using IDT technologies "Designer Tool" software. The selected primers were designed to anneal in exons separated by an intron. Primers were resuspended at 100μM in TE buffer 1X (10 mM Tris-HCl pH8 and 1mM EDTA in ddH2O), diluted at the working concentration of 10μM, and used at the final concentration of 0.5μM.

HIS START PURIFICATION

START domain was amplified from the plasmid STARD3 pLX304 (Harvard Plasmid ID Database) with primers pETSTARD3-bamhI-F: 5'-ATGGATCCGGGGTCTGACAATGAATCAGATG, STARD3-ecoRI-R: 5'-ATGAATTCTCACGCCCGGGCCCCCAG and cloned in the PET vector. The His-START domain was expressed into BL21 (DE3) cells (ThermoFisher, Waltham, MA, USA) and incubated ON in 5 mL of LB/Amp (100 μg/mL). 4 mL of the culture were added to 200 mL of LB/Amp and was grown at 37°C the OD was 1. The culture was then incubated with IPTG (1 mM) for 4 hours at room temperature. The culture was spin down at 6000 rpm for 15 minutes and the pellet resuspended in 5 mL of buffer A (300 mM NaCl, 50 mM NaH_2PO_4, 20 mM Tris HCl and 10 mM betamercaptoetanolo), sonicated for 1 minutes at 30% of power (Bandeline Sonoplus HD2070, MS72) and spin down at 15000 rpm for 10 minutes at 4°C. The lysate was incubated ON at 4°C with beads (cOmplete His-Tag Purification resin; Roche, Basel, Switzerland) previously washed twice at 5000 rpm for 1 minute with buffer B containing 300 mM NaCl and 50 mM NaH_2PO_4. Then, after three wash with buffer B, the protein was eluted in 200 μl of buffer B/40% Glycerol and imidazole (10, 50, 100, 200 and

400 mM). Equal amount of each fractions (5 μL) were loaded in 12% SDS page using BSA as control of the concentration.

BIOCHEMICAL ASSAY

The IC50 assay was performed in 96 multiwell plate in a total volume of 100 μL. 5 μM of His-START protein was added into each well with **VS1** at 200 μM concentration followed by 1:10 serial dilutions. The substrate 3-hexanoyl-NBD Cholesterol (Cayman Chemical, Hamburg, Germany, EU) was diluted in potassium phosphate 2.5 mM and 2 μM tween 20. The samples were incubated at 37°C for 5 minutes and fluorescence was read at 485 and 535 nm.

CELL LINES AND CELL CULTURES.

Human breast cancer cell lines MCF7 and MDA-MB-231, and colorectal cancer cell line HCT116, were obtained from ATCC (Manassas, VA, US). MCF7 and MDA-MB-231 cells were cultured in DMEM high glucose 10% of fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin and not essential amino acids. MCF7 medium was supplemented with 0.01 mg/mL insulin. HCT116 cells were grown in McCoy's 5A medium 10% of fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. All the cells were cultured at 37°C and 5% carbon dioxide. For the knockdown experiments the cells were infected with a control (PLKO) and two knock-down plasmids (sh1: TRCN0000155040 and sh2: TRCN0000150515) from Sigma-Aldrich (St. Louis, MO, USA). Three days after the infection, the cells were collected and utilized for downstream experiments.

IC_{50} DETERMINATION.

One day before treatment, HCT116, MDA MB 231 and MCF7 cell lines were plated into 96 well plates, 500 cells/well. Then, the cells were treated with **VS1** at the concentration of 1 mM followed by 1:2 serial dilutions. After 96 hours, cell viability was evaluated by CellTiter-GLO luminescent assay from Promega (Madison, WI, USA) with Infinite M1000 PRO microplate reader (Tecan, Mannedorf, Switzerland).

STARD3 PROTEIN STABILITY.

$3x10^5$ NIH3T3 cells were plated one day before treatment. Cells were treated with 100 μM of **VS1** and DMSO and after 24 h treated with 10 ug/mL of cycloheximide (CHX). Control and **VS1**-treated cells were collected after 0, 3, 6, 12, 24, 36 hours and analyzed by western blot.

COLONY ASSAY.

300 cells were plated in 6 well plates one day before the treatment. Cells were treated with 0, 50 and 100 μM of **VS1**. The cells were grown for 10 days changing the medium every 2-3 days. Then the cells were fixed with 4% of paraformaldehyde for 5 minutes, stained with crystal violet 0,05% for 30 minutes and washed twice. Each experiment was replicated two times.

WESTERN BLOT ANALYSIS.

Cells were lysed in RIPA buffer 0,1% SDS plus protease (Complete-EDTA-free Protease Inhibitor Cocktail; Roche, Basel, Switzerland) and phosphatase inhibitors (NaF and $NaVO_4$ from Sigma-Aldrich; St. Louis, MO, USA) for 20 minutes on ice. Samples were sonicated for 5 sec. at 10% power (Bandeline Sonoplus HD2070, MS72) and centrifuged at 13,8xg at 4°C for 20 minutes. Equal amount of each protein (50 μg) was loaded in 12% SDS page. Proteins were transferred onto nitrocellulose membranes and blocked for 30 minutes in 5% non-fat

dried milk in TBS 0,1% Tween 20 (TBS-T). The membranes were incubated ON with primary antibodies at 4°C, washed in rotation three times for 10 minutes with TBS-T and incubated with HRP-conjugated secondary antibodies for 1h at room temperature. The results were visualized by ECL western blot analysis detection ChemiDoc imaging system (Bio-Rad, Hercules, CA, USA).

Primary antibodies: mouse anti-Hsp70 (1:1000; cat. no. sc-24), mouse anti-MLN64 (G3) (1:100; cat.no. sc-390040) and goat anti-Vinculin (1:5000; cat.no. sc-7649) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit anti-FAK (1:1000; cat.no. 3285) and rabbit anti-PhosphoFAK (Tyr397) (1:1000; cat.no.3283) from Cell Signaling Technology (Danvers, MA, USA). Secondary antibodies were mouse anti-rabbit IgG (1:5000; cat. no. 31464), goat anti-mouse IgG (1:5000; cat. no. 31432) and anti-goat IgG (1:5000) from ThermoFisher Scientific (Waltham, MA, USA).

CASPASE 3/7 ASSAY.

STARD3 was chemically inhibited with **VS1** in different cell lines. Cell lines were collected and $1x10^5$ cells were lysed in 10 μL of NP-40 buffer and incubated with 10 μL of Caspase-Glo 3/7 assay system from Promega (Madison, WI, US) at room temperature. The results were evaluated by with Infinite M1000 PRO microplate reader (Tecan, Mannedorf, Switzerland).

RT-PCR.

$3x10^5$ NIH3T3 cells were plated and the day after treated with **VS1** at 0,50,100 μM for 48h. The cells were collected and proceed with RNA extraction using the Smarter Nucleic Acid Preparation kit from Smarter Nucleic Acid Sample Preparation kit (Stratec Molecular; Birkenfeld, Germany, EU). 400 ng of total RNA were reverse transcribed in a 10 μL reaction using GoScript Reverse Transcription System kit (Promega, Madison, CA, USA). For the

amplification of the target genes, 1/10 volumes of cDNA reaction was used. For the Semi-quantitative PCR, the DNA was amplified using GoTaq® G2 Polymerase and Master Mix from Promega (Madison, WI, USA). Hprt gene was used as a control of the reaction. The PCR were carried out in a final volume of 20 μL as described in the manufacturer's protocol and the cycles were as follow: 5 min at 95°C; 20 s at 95°C, 30 s at 60°C, 30 s at 72°C x 30 cycles. The products were analyzed via 3% agarose gel electrophoresis.

STATISTICAL ANALYSIS

All the results were the average of at least 3 experiments. The software adopted for the analysis was Microsoft Excel.

BYBLIOGRAPHY

Aebi, S. et al. Resistance to cytotoxic drugs in DNA mismatch repair-deficient cells. *Clin. Cancer Res*. 3, 1763–1767 (1997).

Allegra CJ, Jessup JM, Somerfield MR, Hamilton SR, Hammond EH, Hayes DF, et al. (2009) American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. Journal of clinical oncology: official journal of the American Society of Clinical Oncology. 2009;27(12):2091-6. Epub 2009/02/04.

Allegra CJ, Yothers G, O'Connell MJ, Sharif S, Petrelli NJ, Lopa SH, (2013). Bevacizumab in stage II-III colon cancer: 5-year update of the National Surgical Adjuvant Breast and Bowel Project C-08 trial. *J Clin Oncol*. 2013;31(3):359–364. 10.1200/JCO.2012.44.4711

Alpy F, Rousseau A, Schwab Y, Legueux F, Stoll I, Wendling C, Spiegelhalter C, Kessler P, Mathelin C, Rio MC, Levine TP, Tomasetto C. (2013). STARD3 or STARD3NL and VAP form a novel molecular tether between late endosomes and the ER. *J Cell Sci.* 2013 Dec 1;126(Pt 23):5500-12. doi: 10.1242/jcs.139295. Epub 2013 Oct 8.

Alpy F, Tomasetto C. (2005) Give lipids a START: the StAR-related lipid transfer (START) domain in mammals. *J Cell Sci*. 2005 Jul 1;118(Pt 13):2791-801.

Alpy F., Tomasetto C. (2014) STARD3: A Lipid Transfer Protein in Breast Cancer and Cholesterol Trafficking. In: Clark B., Stocco D. (eds) Cholesterol Transporters of the START Domain Protein Family in Health and Disease. Springer, New York, NY

Al-Sohaily S, Biankin A, Leong R, Kohonen-Corish M, Warusavitarne J (2012) Molecular pathways in colorectal cancer. *J Gastroenterol Hepatol. 2012 Sep; 27(9):1423-31*

American Association for Cancer Research. (2017) First tissue-agnostic drug approval issued. *Cancer Discov.* 2017, 7, 656.

André, T., Boni, C., Navarro, M., Tabernero, J., Hickish, T., Topham, C., Bonetti, A., Clingan, P., Bridgewater, J., Rivera, F., et al. (2009). Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. J. *Clin. Oncol. Off. J. Am. Soc. Clin. Oncol*. *27*, 3109–3116.

Aoki T, Takeda S, Yanagisawa A, Kato Y, Ajioka Y, Watanabe H, Kudo S, Nakamura Y (1994). APC and p53 mutations in de novo colorectal adenocarcinomas. *Hum Mutat.* 1994; 3(4):342-6.

Baba, Y.; Huttenhower, C.; Nosho, K.; Tanaka, N.; Shima, K.; Hazra, A.; Schernhammer, E.S.; Hunter, D.J.; Giovannucci, E.L.; Fuchs, C.S.; et al. (2010) Epigenomic diversity of colorectal cancer indicated by LINE-1 methylation in a database of 869 tumors. *Mol. Cancer* 2010, *9*, 125.

Baker BY, Yaworsky DC, Miller WL. (2005) A pH-dependent molten globule transition is required for activity of the steroidogenic acute regulatory protein, StAR. *Journal of Biological Chemistry* 28041753–41760. doi:10.1074/jbc.M510241200.

Bamford S, Dawson E, Forbes S, Clements J, Pettett R, Dogan A et al. (2004). The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. *Br J Cancer* 91: 355–358.

Barault L, Veyrie N, Jooste V, Lecorre D, Chapusot C, Ferraz JM et al. (2008). Mutations in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in a population-based series of colon cancers. *Int J Cancer* 122: 2255–2259.

Barras D., (2015). BRAF Mutation in Colorectal Cancer: An Update. *Biomark Cancer*. 2015; 7(Suppl 1): 9–12. Published online 2015 Sep 6. doi: 10.4137/BIC.S25248

Beckman RA, Loeb LA (2005). Genetic instability in cancer: theory and experiment. *Semin Cancer Biol*. 2005 Dec; 15(6):423-35.

Benvenuti S, Frattini M, Arena S, Zanon C, Cappelletti V, Coradini D et al. (2008). PIK3CA cancer mutations display gender and tissue specificity patterns. *Hum Mutat* 29: 284–288.

Bettington M, Walker N, Clouston A, Brown I, Leggett B, Whitehall V. (2013) The serrated pathway to colorectal carcinoma: current concepts and challenges. *Histopathology.* 2013 Feb;62(3):367-86. doi: 10.1111/his.12055.

Bienz M, Clevers H. (2000). Linking colorectal cancer to WNT signaling. *Cell.* 2000 Oct 13;103(2):311-20

Bokemeyer C, Bondarenko I, Makhson A, Hartmann JT, Aparicio J, Zampino M et al. (2007). Cetuximab plus 5-FU/FA/oxaliplatin (FOLFOX-4) versus FOLFOX-4 in the first-line treatment of metastatic colorectal cancer (mCRC): OPUS, a randomized phase II study. *Proc Am Soc Clin Oncol* 25: 4035.

Boland, P.M.; Ma, W.W. (2017) Immunotherapy for Colorectal Cancer. *Cancers (Basel)* 2017, *9*, 50.

Borthwick, F., Allen, A.-M., Taylor, J.M., and Graham, A. (2010). Overexpression of STARD3 in human monocyte/macrophages induces an anti-atherogenic lipid phenotype. *Clin. Sci. Lond. Engl.* 1979 119, 265–272.

Bos JL. (1989). ras oncogenes in human cancer: a review. *Cancer Res* 49: 4682–4689.

Bose HS, Whittal RM, Ran Y, Bose M, Baker BY, Miller WL. (2008) StAR-like activity and molten globule behavior of StARD6, a male germ-line protein. *Biochemistry* 472277–2288. doi:10.1021/bi701966a.

Boyle P. and Langman J. (2000) ABC of colorectal cancer: Epidemiology. *BMJ: British Medical Journal* 321(7264), 805.

Briggs, S.; Tomlinson, I. (2013) Germline and somatic polymerase epsilon and delta mutations define a new class of hypermutated colorectal and endometrial cancers. *J. Pathol.* 2013, *230*, 148–153.

Cai W, Ye L, Sun J, Mansel RE, Jiang WG. (2010) Expression of MLN64 influences cellular matrix adhesion of breast cancer cells, the role for focal adhesion kinase. *Int J Mol Med*. 2010 Apr;25(4):573-80.

Cancer Genome Atlas Network (2012). Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 487, 330–337.

Caron KM, Soo SC, Wetsel WC, Stocco DM, Clark BJ, Parker KL. (1997) Targeted disruption of the mouse gene encoding steroidogenic acute regulatory protein provides insights into congenital lipoid adrenal hyperplasia. *Proc Natl Acad Sci U S A.* 1997 Oct 14;94(21):11540-5.

Chen, R.Z.; Pettersson, U.; Beard, C.; Jackson-Grusby, L.; Jaenisch, R. (1998) DNA hypomethylation leads to elevated mutation rates. *Nature* 1998, *395*, 89–93.

Chen, W.; Liu, F.; Ling, Z.; Tong, X.; Xiang, C. (2012) Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. *PLoS ONE* 2012, 7, e39743.

Cheng L., Lopez-Beltran A., Massari F., MacLennan G., Montironi R., (2018). Molecular testing for BRAF mutations to inform melanoma treatment decisions: a move toward precision medicine. *Mod Pathol*. 2018 Jan; 31(1): 24–38. Published online 2017 Nov 17. doi: 10.1038/modpathol.2017.104

Christoph Bock, Jörn Walter, Martina Paulsen, and Thomas Lengauer (2007) CpG Island Mapping by Epigenome Prediction. *PLoS Comput Biol*. 2007 Jun; 3(6): e110.

Chu E (2012) An update on the current and emerging targeted agents in metastatic colorectal cancer. *Clin Colorectal Cancer*. 2012 Mar; 11(1):1-13

Church JM. (2004) Clinical significance of small colorectal polyps. *Dis Colon Rectum*. 2004 Apr;47(4):481-5.

Clark BJ. (2012) The mammalian START domain protein family in lipid transport in health and disease. *J Endocrinol*. 2012 Mar;212(3):257-75. doi: 10.1530/JOE-11-0313. Epub 2011 Sep 30.

Collura, A.; Lagrange, A.; Svrcek, M.; Marisa, L.; Buhard, O.; Guilloux, A.; Wanherdrick, K.; Dorard, C.; Taieb, A.; Saget, A.; et al. (2014) Patients with colorectal tumors with microsatellite instability and large deletions in HSP110 T17 have improved response to 5-fluorouracil-based chemotherapy. *Gastroenterology* 2014, *146*, 401–411.

Corcoran RB, Ebi H, Turke AB, Coffee EM, Nishino M, Cogdill AP, Brown RD, Della Pelle P, Dias-Santagata D, Hung KE, Flaherty KT, Piris A, Wargo JA, Settleman J, Mino-Kenudson M, Engelman JA. (2012) EGFR-mediated re-activation of MAPK signaling contributes to insensitivity of BRAF mutant colorectal cancers to RAF inhibition with vemurafenib. *Cancer Discov.* 2012 Mar;2(3):227-35. doi: 10.1158/2159-8290.CD-11-0341. Epub 2012 Jan 16.

Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C, Chau I, Van Cutsem E. (2004) Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med*. 2004 Jul 22;351(4):337-45.

De Roock, W.; Claes, B.; Bernasconi, D.; De Schutter, J.; Biesmans, B.; Fountzilas, G.; Kalogeras, K.T.; Kotoula, V.; Papamichael, D.; Laurent-Puig, P.; et al. (2010) Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: A retrospective consortium analysis. *Lancet Oncol*. 2010, 11, 753–762.

Derynck, R.; Akhurst, R.J.; Balmain, A. (2001) TGF-beta signaling in tumor suppression and cancer progression. *Nat. Genet.* 2001, *29*, 117–129.

Dienstmann R, Vermeulen L, Guinney J, Kopetz S, Tejpar S, Tabernero J (2017) Consensus molecular subtypes and the evolution of precision medicine in colorectal cancer. *Nat Rev Cancer*. 2017 Mar 23; 17(4):268.

Dorard, C.; de Thonel, A.; Collura, A.; Marisa, L.; Svrcek, M.; Lagrange, A.; Jego, G.; Wanherdrick, K.; Joly, A.L.; Buhard, O.; et al. (2011) Expression of a mutant HSP110 sensitizes colorectal cancer cells to chemotherapy and improves disease prognosis. *Nat. Med.* 2011, *17*, 1283–1289.

Douillard JY, Siena S, Cassidy J, Tabernero J, Burkes R, Barugel M, Humblet Y, Bodoky G, Cunningham D, Jassem J, Rivera F, Kocákova I, Ruff P, Błasińska-Morawiec M, Smakal M, Canon JL, Rother M, Oliner KS, Tian Y, Xu F, Sidhu R: Final results from PRIME: randomized phase III study of panitumumab with FOLFOX4 for first-line treatment of metastatic colorectal cancer. *Ann Oncol* 2014, 25:1346-55.

Dressman MA, Baras A, Malinowski R, Alvis LB, Kwon I, Walz TM, Polymeropoulos MH. (2003) Gene expression profiling detects gene amplification and differentiates tumor types in breast cancer. *Cancer Res*. 2003 May 1;63(9):2194-9.

Ehsan Nazemalhosseini Mojarad, Roya Kishani Farahani, Mahdi Montazer Haghighi, Hamid Asadzadeh Aghdaei, Peter JK Kuppen, and Mohammad Reza Zali. (2013) Clinical implications of BRAF mutation test in colorectal cancer. *Gastroenterol Hepatol Bed Bench.* 2013 Winter; 6(1): 6–13.

Elrasheid A. H. Thibodeau, Nicola Miller, Kah Hoong Chang, Catherine Curran, Emer Hennessey, Margaret Sheehan, and Michael J Kerin (2013) Mismatch repair protein expression in colorectal cancer. *J Gastrointest Oncol*. 2013 Dec; 4(4): 397–408.

Esparza-Perusquía M, Olvera-Sánchez S, Flores-Herrera O, Flores-Herrera H, Guevara-Flores A, Pardo JP1, Espinosa-García MT, Martínez F. (2015) Mitochondrial proteases act on STARD3 to activate progesterone synthesis in human syncytiotrophoblast. *Biochim Biophys Acta*. 2015 Jan;1850(1):107-17. doi: 10.1016/j.bbagen.2014.10.009. Epub 2014 Oct 18.

Farrington SM, Tenesa A, Barnetson R, Wiltshire A, Prendergast J, Porteous M, Campbell H, Dunlop MG. (2005). Germline susceptibility to colorectal cancer due to base-excision repair gene defects. *Am J Hum Genet*. 2005 Jul;77(1):112-9

Faulkner, G.J.; Kimura, Y.; Daub, C.O.; Wani, S.; Plessy, C.; Irvine, K.M.; Schroder, K.; Cloonan, N.; Steptoe, A.L.; Lassmann, T.; et al. (2009) The regulated retrotransposon transcriptome of mammalian cells. *Nat. Genet.* 2009, *41*, 563–571.

Fearon ER, Vogelstein B. (1990) A genetic model for colorectal tumorigenesis. *Cell*. 1990 Jun 1;61(5):759-67.

Fernandez-Peralta AM, Nejda N, Oliart S, Medina V, Azcoita MM, GonzalezAguilera JJ. (2005) Significance of mutations in TGFBR2 and BAX in neoplastic progression and patient outcome in sporadic colorectal tumors with highfrequency microsatellite instability. *Cancer genetics and cytogenetics*. 2005;157(1):18-24. Epub 2005/01/29

Ferrara N (2004) Vascular endothelial growth factor as a target for anticancer therapy. *Oncologist*. 2004; 9 Suppl 1():2-10.

Ferrara N, Hillan KJ, Novotny W (2005) Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. *Biochem Biophys Res Commun*. 2005 Jul 29; 333(2):328-35

Finnberg, N., Gokare, P., and S. El-Deiry, W. (2015). Novel and Emerging Targeted Therapies of Colorectal Cancer. *Curr. Clin. Pharmacol*. 10, 279–298

Flaherty KT, Robert C, Hersey P, Nathan P, Garbe C, Milhem M, Demidov LV, Hassel JC, Rutkowski P, Mohr P, Dummer R, Trefzer U, Larkin JM, Utikal J, Dreno B, Nyakas M, Middleton MR, Becker JC, Casey M, Sherman LJ, Wu FS, Ouellet D, Martin AM, Patel K, Schadendorf D. (2012) Improved survival with MEK inhibition in BRAF-mutated melanoma. *N Engl J Med*. 2012 Jul 12;367(2):107-14. doi: 10.1056/NEJMoa1203421. Epub 2012 Jun 4.

Flores-Martin, J., Rena, V., Angeletti, S., Panzetta-Dutari, G., and Genti-Raimondi, S. (2013). The Lipid Transfer Protein StarD7: Structure, Function, and Regulation. *Int. J. Mol. Sci*. 14, 6170–6186.

Fodde, R., Smits, R., and Clevers, H. (2001). APC, signal transduction and genetic instability in colorectal cancer. *Nat. Rev. Cancer 1*, 55–67.

Folkman J, Klagsbrun M. (1987) Angiogenic factors. *Science.* 1987;235(4787):442–447.

Freddie Bray BSc, Jacques Ferlay, Isabelle Soerjomataram, Rebecca L. Siegel, Lindsey A. Torre, Ahmedin Jemal. (2018) Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *A Cancer Journal for Clinicians* doi: 10.3322/caac.21492

Gao, Z.; Guo, B.; Gao, R.; Zhu, Q.; Qin, H. (2015) Microbiota disbiosis is associated with colorectal cancer. *Front. Microbiol.* 2015.

Gavin, P.G.; Paik, S.; Yothers, G.; Pogue-Geile, K.L. (2013) Colon cancer mutation: Prognosis/prediction–response. *Clin. Cancer Res*. 2013, 19, 1301.

Goldstein, J.L., and Brown, M.S. (2009). The LDL receptor. Arterioscler. Thromb. Vasc. Biol. 29, 431–438.

Gong, J.; Wang, C.; Lee, P.P.; Chu, P.; Fakih, M. (2017) Response to PD-1 Blockade in Microsatellite Stable Metastatic Colorectal Cancer Harboring a POLE Mutation. *J. Natl. Compr. Cancer Netw.*2017, *15*, 142–147.

Grady WM, Carethers JM (2008). Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology*. 2008 Oct; 135(4):1079-99.

Gray R, Barnwell J, McConkey C, Hills RK, Williams NS, Kerr DJ (2007) Adjuvant chemotherapy versus observation in patients with colorectal cancer: a randomised study. *Lancet* 2007, 370:2020-9.

Guinney J, Dienstmann R, Wang X, de Reyniès A, Schlicker A, Soneson C, Marisa L, Roepman P, Nyamundanda G, Angelino P, Bot BM, Morris JS, Simon IM, Gerster S, Fessler E, De Sousa E Melo F, Missiaglia E, Ramay H, Barras D, Homicsko K, Maru D, Manyam GC, Broom B, Boige V, Perez-Villamil B, Laderas T, Salazar R, Gray JW, Hanahan D, Tabernero J, Bernards R, Friend SH, Laurent-Puig P, Medema JP,

Sadanandam A, Wessels L, Delorenzi M, Kopetz S, Vermeulen L, Tejpar S (2015) The consensus molecular subtypes of colorectal cancer. *Nat Med.* 2015 Nov; 21(11):1350-6

Haller DG, Tabernero J, Maroun J, Braud F de, Price T, van Cutsem E, Hill M, Gilberg F, Rittweger K, Schmoll H: Capecitabine plus oxaliplatin compared with fluorouracil and folinic acid as adjuvant therapy for stage III colon cancer. *J Clin Oncol* 2011, 29:1465-71

Hamilton, S.R., Aaltonen, L.A., Cancer, I.A. for R. on, Organization, W.H., and others (2000). Pathology and genetics of tumours of the digestive system (IARC press Lyon:).

Heinemann V, Stintzing S, Kirchner T, Boeck S, Jung A. (2009) Clinical relevance of EGFR- and KRAS-status in colorectal cancer patients treated with monoclonal antibodies directed against the EGFR. *Cancer Treat Rev.* 2009 May;35(3):262-71. doi: 10.1016/j.ctrv.2008.11.005. Epub 2008 Dec 30.

Horibata, Y., and Sugimoto, H. (2010). StarD7 mediates the intracellular trafficking of phosphatidylcholine to mitochondria. J. Biol. Chem. 285, 7358–7365.

Howlander, n, Noone, A., Krapcho, M., Garshell, J., Miller, D., Altekruse, S., Kosary, C., Yu, M., Ruhl, J., Tatalovich, Z., et al. (2014). SEER Cancer Statistics Review, 1975-2012.

Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med.* 2004 Jun 3; 350(23):2335-42.

Huxley, R.R., Ansary-Moghaddam, A., Clifton, P., Czernichow, S., Parr, C.L., and Woodward, M. (2009). The impact of dietary and lifestyle risk factors on risk of colorectal cancer: A quantitative overview of the epidemiological evidence. *Int. J. Cancer 125*, 171–180.

Iacopetta B. (2003) TP53 mutation in colorectal cancer. Hum Mutat. 2003;21:271–276

Imai, K. & Yamamoto, H. Carcinogenesis and microsatellite instability: the interrelationship between genetics and epigenetics. Carcinogenesis 29, 673–680 (2008).

Inamura, K.; Yamauchi, M.; Nishihara, R.; Kim, S.A.; Mima, K.; Sukawa, Y.; Li, T.; Yasunari, M.; Zhang, X.; Wu, K.; et al. (2015) Prognostic significance and molecular features of signet-ring cell and mucinous components in colorectal carcinoma. *Ann. Surg. Oncol*. 2015, 22, 1226–1235.

Inamura, K.; Yamauchi, M.; Nishihara, R.; Lochhead, P.; Qian, Z.R.; Kuchiba, A.; Kim, S.A.; Mima, K.; Sukawa, Y.; Jung, S.; et al. (2014) Tumor LINE-1 methylation level and microsatellite instability in relation to colorectal cancer prognosis. *J. Natl. Cancer Inst.* 2014, *106*.

Iyer LM, Koonin EV, Aravind L. (2001) Adaptations of the helix-grip fold for ligand binding and catalysis in the START domain superfamily. *Proteins* 43134–144. doi:10.1002/1097-0134(20010501)43:2<134::AID-PROT1025>3.0.CO;2-I.

Jang, Bum Jun Kim, Jung Han Kim, Hyeong Su Kim (2017). The addition of bevacizumab in the first-line treatment for metastatic colorectal cancer: an updated meta-analysis of randomized trials. *Oncotarget*. 2017 Sep 22; 8(42): 73009–73016. doi: 10.18632/oncotarget.20314

Jass JR (2007) Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology.* 2007 Jan; 50(1):113-30.

Jeremy R Jass. (2006) Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology* Volume 50, Issue 1 January 2007, Pages 113-130

Jia, M.; Gao, X.; Zhang, Y.; Hoffmeister, M.; Brenner, H. (2016) Different definitions of CpG island methylator phenotype and outcomes of colorectal cancer: A systematic review. *Clin. Epigenet*. 2016, 8, 25.

Jones, R.P.; Sutton, P.A.; Evans, J.P.; Clifford, R.; McAvoy, A.; Lewis, J.; Rousseau, A.; Mountford, R.; McWhirter, D.; Malik, H.Z. (2017) Specific mutations in KRAS codon 12 are associated with worse overall survival in patients with advanced and recurrent colorectal cancer. *Br. J. Cancer* 2017, 116, 923–929.

Kane MF, Loda M, Gaida GM, et al. Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res* 1997;57:808-11

Kanno, K., Wu, M.K., Scapa, E.F., Roderick, S.L., and Cohen, D.E. (2007). Structure and function of phosphatidylcholine transfer protein (PC-TP)/StarD2. *Biochim. Biophys. Acta* 1771, 654–662.

Kao J, Pollack JR. (2006) RNA interference-based functional dissection of the 17q12 amplicon in breast cancer reveals contribution of coamplified genes. *Genes Chromosomes Cancer*. 2006 Aug;45(8):761-9.

Kapiteijn E, Putter H, van de Velde CJ. (2002) Impact of the introduction and training of total mesorectal excision on recurrence and survival in rectal cancer in The Netherlands. *The British journal of surgery*. 2002;89(9):1142-9. Epub 2002/08/23.

Kather, J.N.; Poleszczuk, J.; Suarez-Carmona, M.; Krisam, J.; Charoentong, P.; Valous, N.A.; Weis, C.A.; Tavernar, L.; Leiss, F.; Herpel, E.; et al. (2017) In silico modeling of immunotherapy and stroma-targeting therapies in human colorectal cancer. *Cancer Res.* 2017, *77*, 6442–6452.

Katz, L.H.; Likhter, M.; Jogunoori, W.; Belkin, M.; Ohshiro, K.; Mishra, L. (2016) TGF-beta signaling in liver and gastrointestinal cancers. *Cancer Lett.* 2016, *379*, 166–172.

Kawakami, H.; Zaanan, A.; Sinicrope, F.A. (2015) Microsatellite instability testing and its role in the management of colorectal cancer. *Curr. Treat. Options Oncol.* 2015, *16*, 30.

Kawai, K., Kiyota, M., Seike, J., Deki, Y., and Yagisawa, H. (2007). START-GAP3/DLC3 is a GAP for RhoA and Cdc42 and is localized in focal adhesions regulating cell morphology. *Biochem. Biophys. Res. Commun*. 364, 783–789.

Kim IJ, Kang HC, Park JH, Shin Y, Ku JL, Lim SB, et al. (2003) Development and applications of a beta-catenin oligonucleotide microarray: beta-catenin mutations are dominantly found in the proximal colon cancers with microsatellite instability. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2003;9(8):2920-5. Epub 2003/08/13

Kim JH, Kang GH. (2014) Molecular and prognostic heterogeneity of microsatellite unstable colorectal cancer. *World journal of gastroenterology: WJG*. 2014;20(15):4230-43. Epub 2014/04/26

King SR, Bhangoo A, Stocco DM. (2011) Functional and physiological consequences of StAR deficiency: role in lipoid congenital adrenal hyperplasia. *Endocr Dev*. 2011;20:47-53. doi: 10.1159/000321214. Epub 2010 Dec 16.

Kirkby, B., Roman, N., Kobe, B., Kellie, S., and Forwood, J.K. (2010). Functional and structural properties of mammalian acyl-coenzyme A thioesterases. *Prog. Lipid Res*. 49, 366–377.

Kishida, T., Kostetskii, I., Zhang, Z., Martinez, F., Liu, P., Walkley, S.U., Dwyer, N.K., Blanchette- Mackie, E.J., Radice, G.L., and Strauss, J.F. (2004). Targeted mutation of the MLN64 START domain causes only modest alterations in cellular sterol metabolism. *J. Biol. Chem*. 279, 19276–19285.

Kopetz S, Chang GJ, Overman MJ, Eng C, Sargent DJ, Larson DW, Grothey A, Vauthey JN, Nagorney DM, McWilliams RR. (2009) Improved survival in metastatic colorectal cancer is associated with adoption of hepatic resection and improved chemotherapy. *J Clin Oncol*. 2009 Aug 1;27(22):3677-83. Doi10.1200/JCO.2008.20.5278

Ilic, Slobodan Jankovic, Milena Ilic (2016). Bevacizumab Combined with Chemotherapy Improves Survival for Patients with Metastatic Colorectal Cancer: Evidence from Meta Analysis. *PLoS One*. 2016; 11(8): e0161912. doi: 10.1371/journal.pone.0161912

Lamy PJ, Fina F, Bascoul-Mollevi C, Laberenne AC, Martin PM, Ouafik L, Jacot W. (2011) Quantification and clinical relevance of gene amplification at chromosome 17q12-q21 in human epidermal growth factor receptor 2-amplified breast cancers. *Breast Cancer Res*. 2011 Feb 2;13(1):R15. doi: 10.1186/bcr2824.

Lavecchia A, Di Giovanni C. (2013) Virtual screening strategies in drug discovery: a critical review. *Curr. Med. Chem*. 2013;20(23):2839–2860. doi: 10.2174/09298673113209990001.

Lee, D.W.; Han, S.W.; Cha, Y.; Bae, J.M.; Kim, H.P.; Lyu, J.; Han, H.; Kim, H.; Jang, H.; Bang, D.; et al. (2017) Association between mutations of critical pathway genes and survival outcomes according to the tumor location in colorectal cancer. *Cancer* **2017**, *123*, 3513–3523.

Létourneau, D., Lorin, A., Lefebvre, A., Frappier, V., Gaudreault, F., Najmanovich, R., Lavigne, P., and LeHoux, J.-G. (2012). StAR-related lipid transfer domain protein 5 binds primary bile acids. *J. Lipid Res*. 53, 2677–2689.

Liapis A, Chen FW, Davies JP, Wang R, Ioannou YA (2012) MLN64 Transport to the Late Endosome Is Regulated by Binding to 14-3-3 via a Non-canonical Binding Site. *PLoS ONE* 7(4): e34424. https://doi.org/10.1371/journal.pone.0034424

Liefers GJ, Tollenaar RA.(2002) Cancer genetics and their application to individualised medicine. *Eur J Cancer*. 2002;38(7):872-9. Epub 2002/04/30.

Lievre A, Blons H, Laurent-Puig P. (2010) Oncogenic mutations as predictive factors in colorectal cancer. *Oncogene*. 2010;29(21):3033-43. Epub 2010/04/13

Lievre, A., Bachet, J.-B., Le Corre, D., Boige, V., Landi, B., Emile, J.-F., Côté, J.-F., Tomasic, G., Penna, C., Ducreux, M., et al. (2006). KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res*. *66*, 3992–3995.

Liu B, Parsons R, Papadopoulos N, et al. Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. *Nat Med* 1996;2:169-74

Long, M. J. C.; Gollapalli, D. R.; Hedstrom, L. (2012) Inhibitor Mediated Protein Degradation. *Chem. Biol*. 2012, 19 (5), 629– 637.

Lu, Y.; Chen, J.; Zheng, J.; Hu, G.; Wang, J.; Huang, C.; Lou, L.; Wang, X.; Zeng, Y. (2016) Mucosal adherent bacterial dysbiosis in patients with colorectal adenomas. *Sci. Rep*. 2016, *6*, 26337.

Lucia Pérez-Cabornero, Mar Infante Sanz, Eladio Velasco Sampedro, Enrique Lastra Aras, Alberto Acedo Becares, Cristina Miner Pino and Mercedes Durán Domínguez. (2011) Frequency of Rearrangements in Lynch Syndrome Cases Associated with MSH2: Characterization of a New Deletion Involving both EPCAM and the 5′ Part of MSH2. *Cancer Prevention Research* Volume 4, Issue 10

Lukasik, D., Wilczek, E., Wasiutynski, A., and Gornicka, B. (2011). Deleted in liver cancer protein family in human malignancies (Review). *Oncol. Lett*. 2, 763–768.

Luke JJ, Flaherty KT, Ribas A, Long GV, (2017). Targeted agents and immunotherapies: optimizing outcomes in melanoma. *Nat Rev Clin Oncol*. 2017 Aug;14(8):463-482. doi: 10.1038/nrclinonc.2017.43. Epub 2017 Apr 4.

Lynch HT, de la Chapelle A. (2003) Hereditary colorectal cancer. *N Engl J Med*. 2003;348(10):919-32. Epub 2003/03/07.

Marra G, Boland CR. Hereditary nonpolyposis colorectal cancer: the syndrome, the genes, and historical perspectives. *J Natl Cancer Inst* 1995;87:1114-25

Marshall CJ. (1996). Ras effectors. *Curr Opin Cell Biol* 8: 197–204

Masugi Y, Nishihara R, Yang J, et al (2017) Tumour CD274 (PD-L1) expression and T cells in colorectal cancer *Gut* 2017;66:1463-1473.

Maxfield FR, van Meer G. (2010) Cholesterol, the central lipid of mammalian cells. *Current Opinion in Cell Biology* 22422–429. doi:10.1016/j.ceb.2010.05.004.

McCormick F (2015). KRAS as a therapeutic target. *Clin Cancer Res*. 2015;21:1797–801

McLean GW, Carragher NO, Avizienyte E, Evans J, Brunton VG, Frame MC. (2005) The role of focal-adhesion kinase in cancer - a new therapeutic opportunity. *Nat Rev Cancer.* 2005;5:505–515. doi: 10.1038/nrc1647.

Mesmin, B., Pipalia, N.H., Lund, F.W., Ramlall, T.F., Sokolov, A., Eliezer, D., and Maxfield, F.R. (2011). STARD4 abundance regulates sterol transport and sensing. Mol. Biol. Cell 22, 4004 4015.

Michelle C. Mendoza, E. Emrah Er, and John Blenis (2011) The Ras-ERK and PI3K-mTOR Pathways: Cross-talk and Compensation. *Trends Biochem Sci.* 2011 Jun; 36(6): 320–328.

Minamoto T, Sawaguchi K, Mai M, Yamashita N, Sugimura T, Esumi H (1994). Infrequent K-ras activation in superficial-type (flat) colorectal adenomas and adenocarcinomas. *Cancer Res*. 1994 Jun 1; 54(11):2841-4.

Miyaki M, Iijima T, Kimura J, et al. (1999) Frequent mutation of beta-catenin and APC genes in primary colorectal tumors from patients with hereditary nonpolyposis colorectal cancer. *Cancer Res*; 59:4506–9

Montero J1, Morales A, Llacuna L, Lluis JM, Terrones O, Basañez G, Antonsson B, Prieto J, García-Ruiz C, Colell A, Fernández-Checa JC. (2008) Mitochondrial cholesterol contributes to chemotherapy resistance in hepatocellular carcinoma. *Cancer Res*. 2008 Jul 1;68(13):5246-56. doi: 10.1158/0008-5472.CAN-07-6161.

Mueller, C.; Aschacher, T.; Wolf, B.; Bergmann, M. (2018) A role of LINE-1 in telomere regulation. *Front. Biosci.*2018, *23*, 1310–1319.

Nagai, Y.; Sunami, E.; Yamamoto, Y.; Hata, K.; Okada, S.; Murono, K.; Yasuda, K.; Otani, K.; Nishikawa, T.; Tanaka, T.; et al. (2017) LINE-1 hypomethylation status of circulating cell-free DNA in plasma as a biomarker for colorectal cancer. *Oncotarget* 2017, *8*, 11906–11916.

Noffsinger AE (2009). Serrated polyps and colorectal cancer: new pathway to malignancy. *Annu Rev Pathol*. 2009;4:343-64. doi: 10.1146/annurev.pathol.4.110807.092317.

Ogino S, Nosho K, Kirkner GJ, Kawasaki T, Meyerhardt JA, Loda M, et al. (2009) CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. *Gut*. 2009;58(1):90-6. Epub 2008/10/04.

Overman MJ, McDermott R, Leach JL, Lonardi S, Lenz HJ, Morse MA, Desai J, Hill A, Axelson M, Moss RA, Goldberg MV, Cao ZA, Ledeine JM, Maglinte GA, Kopetz S, André T (2015) Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): an open-label, multicentre, phase 2 study. *Lancet Oncol*. 2017 Sep;18(9):1182-1191. doi: 10.1016/S1470-2045(17)30422-9. Epub 2017 Jul 19.

Pancione M, Remo A, Colantuoni V. (2002) Genetic and epigenetic events generate multiple pathways in colorectal cancer progression. *Patholog Res Int.* 2012; 2012():509348.

Parsons DW, Wang TL, Samuels Y, Bardelli A, Cummins JM, DeLong L et al. (2005). Colorectal cancer: mutations in a signalling pathway. *Nature* 436: 792.

Petrelli N, Douglass HO, Herrera L, Russell D, Stablein DM, Bruckner HW, Mayer RJ, Schinella R, Green MD, Muggia FM: The modulation of fluorouracil with leucovorin in metastatic colorectal carcinoma: a prospective randomized phase III trial. Gastrointestinal Tumor Study Group. *J Clin Oncol* 1989, 7:1419-26.

Pino MS, Chung DC. (2010) The chromosomal instability pathway in colon cancer. *Gastroenterology*. 2010 Jun;138(6):2059-72. doi: 10.1053/j.gastro.2009.12.065.

Piñol V, Castells A, Andreu M, Castellví-Bel S, Alenda C, Llor X, Xicola RM, Rodríguez-Moranta F, Payá A, Jover R, Bessa X; Gastrointestinal Oncology Group of the Spanish Gastroenterological Association. (2005). Accuracy of revised Bethesda guidelines, microsatellite instability, and immunohistochemistry for the identification of patients with hereditary nonpolyposis colorectal cancer. JAMA. 2005 Apr 27;293(16):1986-94.

Ponting CP, Aravind L (1999) START: a lipid-binding domain in StAR, HD-ZIP and signalling proteins. *Trends in Biochemical Sciences* 24130–132. doi:10.1016/S0968-0004(99)01362-6.

Popanda, O.; Zheng, C.; Magdeburg, J.R.; Buttner, J.; Flohr, T.; Hagmuller, E.; Thielmann, H.W. (2000) Mutation analysis of replicative genes encoding the large subunits of DNA polymerase alpha and replication factors A and C in human sporadic colorectal cancers. *Int. J. Cancer* 2000, *86*, 318–324.

Popat, S., Hubner, R. & Houlston, R. S. Systematic review of microsatellite instability and colorectal cancer prognosis. J. Clin. Oncol. 23, 609–618 (2005).

Manuela Porru, Luca Pompili, Carla Caruso, Annamaria Biroccio and Carlo Leonetti. (2018) Targeting KRAS in metastatic colorectal cancer: current strategies and emerging opportunities. *J Exp Clin Cancer Res*. 2018; 37: 57. Published online 2018 Mar 13. doi: [10.1186/s13046-018-0719-1]

Prenen, H., Vecchione, L., and Van Cutsem, E. (2013). Role of targeted agents in metastatic colorectal cancer. *Target. Oncol*. *8*, 83–96.

Qin C., Nagao T., Grosheva I., Maxfield FR, Pierini LM. (2006) Elevated plasma membrane cholesterol content alters macrophage signaling and function. 26372–378. doi:10.1161/01. ATV.0000197848.67999.e1.

Qiu Y, Zhang ZY, Du WD, Ye L, Xu S, Zuo XB, Zhou FS, Chen G, Ma XL, Schneider ME, Xia HZ, Zhou Y, Wu JF, Yuan-Hong X, Zhang XJ. (2014) Association analysis of ERBB2 amplicon genetic polymorphisms and STARD3 expression with risk of gastric cancer in the Chinese population. *Gene*. 2014 Feb 10;535(2):225-32. doi: 10.1016/j.gene.2013.11.030. Epub 2013 Nov 28.

Reddy A.S, Pati S.P, Kumar P.P, Pradeep H.N, Sastry G.N. (2007) Virtual screening in drug discovery — a computational perspective. *Curr. Protein Pept. Sci*. 2007;8(4):329–351. doi: 10.2174/138920307781369427.

Remick, S. C. et al. Phase I trial of 5-fluorouracil and dipyridamole administered by seventy-two-hour concurrent continuous infusion. *Cancer Res.* 50, 2667–2672 (1990).

Ribic, C.M., Sargent, D.J., Moore, M.J., Thibodeau, S.N., French, A.J., Goldberg, R.M., Hamilton, S.R., Laurent-Puig, P., Gryfe, R., Shepherd, L.E., et al. (2003). Tumor microsatellite instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. *N. Engl. J. Med*. 349, 247–257.

Richard Boland, Ajay Goel. (2010) Microsatellite Instability in Colorectal Cancer. *Gastroenterology* Volume 138, Issue 6, Pages 2073–2087.e3

Rodic, N.; Burns, K.H. (2013) Long interspersed element-1 (LINE-1): Passenger or driver in human neoplasms? *PLoS Genet.* 2013, *9*, e1003402.

Rodrigues, N.R., Rowan, A., Smith, M.E., Kerr, I.B., Bodmer, W.F., Gannon, J.V., and Lane, D.P. (1990). p53 mutations in colorectal cancer. *Proc. Natl. Acad. Sci*. U. S. A. *87*, 7555–7559.

Rodriguez-Agudo, D., Ren, S., Wong, E., Marques, D., Redford, K., Gil, G., Hylemon, P., and Pandak, W.M. (2008). Intracellular cholesterol transporter StarD4 binds free cholesterol and increases cholesteryl ester formation. *J. Lipid Res*. 49, 1409–1419.

Romanowski MJ, Soccio RE, Breslow JL, Burley SK. (2002) Crystal structure of the Mus musculus cholesterol-regulated START protein 4 (StarD4) containing a StAR-related lipid transfer domain. *PNAS* 996949–6954. doi:10.1073/pnas.052140699.

Rougier P, Mitry E. (2003) Epidemiology, treatment and chemoprevention in colorectal cancer. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*. 2003;14 Suppl 2:ii3-5. Epub 2003/06/18

Russo A, Bazan V, Iacopetta B, Kerr D, Soussi T, Gebbia N; TP53-CRC Collaborative Study Group. (2005) The TP53 colorectal cancer international collaborative study on the prognostic and predictive significance of p53 mutation: influence of tumor site, type of mutation, and adjuvant treatment. *J Clin Oncol*. 2005 Oct 20;23(30):7518-28. Epub 2005 Sep 19

Russo Spena, C.; De Stefano, L.; Palazzolo, S.; Salis, B.; Granchi, C.; Minutolo, F.; Tuccinardi, T.; Fratamico, R.; Crotti, S.; D'Aronco, S.; et al. (2018) Liposomal Delivery of a Pin1 Inhibitor Complexed with Cyclodextrins as New Therapy for High-Grade Serous Ovarian Cancer. *J. Control. Release* 2018, 281, 1–10.

Sahlberg KK, Hongisto V, Edgren H, Mäkelä R, Hellström K, Due EU, Moen Vollan HK, Sahlberg N, Wolf M, Børresen-Dale AL, Perälä M, Kallioniemi O. (2013) The HER2 amplicon includes several genes required for the growth and survival of HER2 positive breast cancer cells. *Mol Oncol*. 2013 Jun;7(3):392-401. doi: 10.1016/j.molonc.2012.10.012. Epub 2012 Nov 24.

Saltz LB, Clarke S, Díaz-Rubio E, Scheithauer W, Figer A, Wong R, Koski S, Lichinitser M, Yang T, Rivera F, Couture F, Sirzén F, Cassidy J: Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy inmetastatic colorectal cancer: a randomized phase III study. *J Clin Oncol* 2008, 26:2013-9.

Samant RS, Shevde LA. (2011) Recent advances in anti-angiogenic therapy of cancer. *Oncotarget*. 2011;2(3):122–134.

Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S et al. (2004). High frequency of mutations of the PIK3CA gene in human cancers. *Science* 304: 554.

Sargent DJ, Goldberg RM, Jacobson SD, Macdonald JS, Labianca R, Haller DG, Shepherd LE, Seitz JF, Francini G: A pooled analysis of adjuvant chemotherapy for resected colon cancer in elderly patients. *N Engl J Med* 2001, 345:1091-7.

Sargent DJ, Marsoni S, Monges G, Thibodeau SN, Labianca R, Hamilton SR, French AJ, Kabat B, Foster NR, Torri V, Ribic C, Grothey A, Moore M, Zaniboni A, Seitz J, Sinicrope F, Gallinger S (2010) Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. *J Clin Oncol* 2010, 28:3219-26.

Satya Narayan and Deodutta Roy.(2003). Role of APC and DNA mismatch repair genes in the development of colorectal cancers. *Mol Cancer.* 2003; 2: 41.

Schmoll HJ, Van Cutsem E, Stein A, Valentini V, Glimelius B, Haustermans K, et al. (2012) ESMO Consensus Guidelines for management of patients with colon and rectal cancer. a personalized approach to clinical decision making. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*. 2012;23(10):2479-516. Epub 2012/09/27.

Schwabe, R.F.; Jobin, C. The microbiome and cancer. Nat. Rev. *Cancer* 2013, 13, 800–812.

Shen L, Toyota M, Kondo Y, Lin E, Zhang L, Guo Y, Hernandez NS, Chen X, Ahmed S, Konishi K, Hamilton SR, Issa JP. (2007) Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. *Proc Natl Acad Sci U S A.* 2007 Nov 20;104(47):18654-9. Epub 2007 Nov 14.

Siegel RL, Miller KD, Jemal A. (2015) Cancer statistics, 2015. CA Cancer J Clin. 2015 Jan-Feb;65(1):5-29. doi: 10.3322/caac.21254

Silva AL, Dawson SN, Arends MJ, Guttula K, Hall N, Cameron EA, Huang TH, Brenton JD, Tavaré S, Bienz M, Ibrahim AE. (2014). Boosting WNT activity during colorectal cancer progression through selective hypermethylation of WNT signaling antagonists. *BMC Cancer*. 2014 Nov 29;14:891. doi: 10.1186/1471-2407-14-891.

Smith G, Carey FA, Beattie J, Wilkie MJ, Lightfoot TJ, Coxhead J, Garner RC, Steele RJ, Wolf CR. (2002). Mutations in APC, Kirsten-ras, and p53--alternative genetic pathways to colorectal cancer. *Proc Natl Acad Sci U S A*. 2002 Jul 9; 99(14):9433-8.

Soccio RE, Adams RM, Romanowski MJ, Sehayek E, Burley SK, Breslow JL. (2002) The cholesterol-regulated StarD4 gene encodes a StAR-related lipid transfer protein with two closely related homologues, StarD5 and StarD6. *PNAS* 996943–6948. doi:10.1073/pnas.052143799.

Soccio RE, Breslow JL. (2003) StAR-related lipid transfer (START) proteins: mediators of intracellular lipid metabolism. *Journal of Biological Chemistry* 27822183–22186. doi:10.1074/jbc.R300003200.

Stephanie A Cohen and Anna Leininger (2014). The genetic basis of Lynch syndrome and its implications for clinical practice and risk management. *Appl Clin Genet*. 2014; 7: 147–158.

Steven M. Powell, Nathan Zilz, Yasmin Beazer-Barclay, Tracy M. Bryan, Stanley R. Hamilton, Stephen N. Thibodeau, Bert Vogelstein & Kenneth W. Kinzler. (1992) APC mutations occur early during colorectal tumorigenesis. *Nature* volume 359, pages 235–237

Stigliano A, Gandini O, Cerquetti L, Gazzaniga P, Misiti S, Monti S, Gradilone A, Falasca P, Poggi M, Brunetti E, Aglianò AM, Toscano V. (2007) Increased metastatic lymph node 64 and CYP17 expression are associated with high stage prostate cancer. *J Endocrinol*. 2007 Jul;194(1):55-61.

Stintzing S. (2014). Management of colorectal cancer. *F1000Prime Rep*. 2014 Nov 4;6:108. doi: 10.12703/P6-108. PMID: 25580262

Takayama T, Miyanishi K, Hayashi T, Sato Y, Niitsu Y (2006) Colorectal cancer: genetics of development and metastasis. *J Gastroenterol*. 2006 Mar; 41(3):185-92.

Thanki K, Nicholls ME, Gajjar A, Senagore AJ, Qiu S, Szabo C, Hellmich MR, Chao C. (2007) Consensus Molecular Subtypes of Colorectal Cancer and their Clinical Implications. *Int Biol Biomed J*. 2017 Summer;3(3):105-111. Epub 2017 Jun 13.

Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. *Science* 1993;260:816-9

Thorstensen L, Lind GE, Lovig T, Diep CB, Meling GI, Rognum TO, et al. (2005) Genetic and epigenetic changes of components affecting the WNT pathway in colorectal carcinomas stratified by microsatellite instability. *Neoplasia*. 2005;7(2):99-108. Epub 2005/04/02

Tol J, Nagtegaal ID, Punt CJ. (2009) BRAF mutation in metastatic colorectal cancer. *N Engl J Med*. 2009;361:98–9.

Tol J, Punt CJ (2010) Monoclonal antibodies in the treatment of metastatic colorectal cancer: a review. *Clin Ther*. 2010 Mar; 32(3):437-53.

Torres, J.Z., Summers, M.K., Peterson, D., Brauer, M.J., Lee, J., Senese, S., Gholkar, A.A., Lo, Y.-C., Lei, X., Jung, K., et al. (2011). The STARD9/Kif16a Kinesin Associates with Mitotic Microtubules and Regulates Spindle Pole Assembly. *Cell* 147, 1309–1323.

Tournigand C, André T, Achille E, Lledo G, Flesh M, Mery-Mignard D, Quinaux E, Couteau C, Buyse M, Ganem G, Landi B, Colin P, Louvet C, Gramont A de: FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study. *J Clin Oncol* 2004, 22:229-37.

Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP (1999) CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci U S A*. 1999 Jul 20; 96(15):8681-6.

Tran, E.; Robbins, P.F.; Lu, Y.C.; Prickett, T.D.; Gartner, J.J.; Jia, L.; Pasetto, A.; Zheng, Z.; Ray, S.; Groh, E.M.; et al. (2016) T-Cell Transfer Therapy Targeting Mutant KRAS in Cancer. *N. Engl. J. Med.* 2016, *375*, 2255–2262.

Tsujishita Y, Hurley JH (2000). Structure and lipid transport mechanism of a StAR-related domain. *Nature Structural Biology* May 2000, 7(5):408-414]

Tsujishita Y1, Hurley JH. (2000) Structure and lipid transport mechanism of a StAR-related domain. *Nat Struct Biol*. 2000 May;7(5):408-14.

Uhlen M, Zhang C, Lee S, Sjöstedt E, Fagerberg L, Bidkhori G, Benfeitas R, Arif M, Liu Z, Edfors F, Sanli K, von Feilitzen K, Oksvold P, Lundberg E, Hober S, Nilsson P, Mattsson J, Schwenk JM, Brunnström H, Glimelius B, Sjöblom T, Edqvist PH, Djureinovic D, Micke P, Lindskog C, Mardinoglu A, Ponten F. (2017) A pathology atlas of the human cancer transcriptome. *Science*. 2017 Aug 18;357(6352). pii: eaan2507. doi: 10.1126/science.aan2507.

Umetani N, Sasaki S, Masaki T, Watanabe T, Matsuda K, Muto T. (2000) Involvement of APC and K-ras mutation in non-polypoid colorectal tumorigenesis. *Br J Cancer*. 2000 Jan; 82(1):9-15.

Van Brummelen, E.M.J.; de Boer, A.; Beijnen, J.H.; Schellens, J.H.M. (2017) BRAF mutations as predictive biomarker for response to anti-EGFR monoclonal antibodies. *Oncologist* 2017, 22, 864–872.

Van Cutsem E, Köhne C, Láng I, Folprecht G, Nowacki MP, Cascinu S, Shchepotin I, Maurel J, Cunningham D, Tejpar S, Schlichting M, Zubel A, Celik I, Rougier P, Ciardiello F: Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. *J Clin Oncol* 2011, 29:2011-9.

Van Cutsem E, Nowacki M, Lang I, Cascinu S, Shchepotin I, Maurel J et al. (2007). Randomized phase III study of irinotecan and 5-FU/ FA with or without cetuximab in the first-line treatment of patients with metastatic colorectal cancer (mCRC): the CRYSTAL trial. *Proc Am Soc Clin Oncol* 25: 4000.

van Nimwegen MJ, van de Water B (2007) Focal adhesion kinase: a potential target in cancer therapy. *Biochem Pharmacol*. 2007 Mar 1; 73(5):597-609.

Vassilev B, Sihto H, Li S, Hölttä-Vuori M, Ilola J, Lundin J, Isola J, Kellokumpu-Lehtinen PL, Joensuu H, Ikonen E. (2015) Elevated levels of StAR-related lipid transfer protein 3 alter cholesterol balance and adhesiveness of breast cancer cells: potential mechanisms contributing to progression of HER2-positive breast cancers. *Am J Pathol*. 2015 Apr;185(4):987-1000. doi: 10.1016/j.ajpath.2014.12.018. Epub 2015 Feb 12.

Veigl ML, Kasturi L, Olechnowicz J, et al. Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancers. *Proc Natl Acad Sci U S A* 1998;95:8698-702

Velho S, Oliveira C, Ferreira A, Ferreira AC, Suriano G, Schwartz Jr S et al. (2005). The prevalence of PIK3CA mutations in gastric and colon cancer. *Eur J Cancer* 41: 1649–1654.

Vetter IR, Wittinghofer A. (2001) The guanine nucleotide-binding switch in three dimensions. *Science.* 2001 Nov 9;294(5545):1299-304.

Vilar, E.; Gruber, S.B. (2010) Microsatellite instability in colorectal cancer-the stable evidence. *Nat. Rev. Clin. Oncol.* 2010, *7*, 153–162.

Vinatzer U, Dampier B, Streubel B, Pacher M, Seewald MJ, Stratowa C, Kaserer K, Schreiber M. (2005) Expression of HER2 and the coamplified genes GRB7 and MLN64 in human breast cancer: quantitative real-time reverse transcription-PCR as a diagnostic alternative to immunohistochemistry and fluorescence in situ hybridization. *Clin Cancer Res*. 2005 Dec 1;11(23):8348-57.

Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL. (1988) Genetic alterations during colorectal-tumor development. *N Engl J Med*. 1988 Sep 1;319(9):525-32.

Watari H, Arakane F, Moog-Lutz C, Kallen CB, Tomasetto C, Gerton GL, Rio MC, Baker ME, Strauss JF 3rd. (1997) MLN64 contains a domain with homology to the steroidogenic acute regulatory protein (StAR) that stimulates steroidogenesis. *Proc Natl Acad Sci U S A*. 1997 Aug 5;94(16):8462-7.

Wilhelm LP, Wendling C, Védie B, Kobayashi T, Chenard MP, Tomasetto C, Drin G, Alpy F. (2017) STARD3 mediates endoplasmic reticulum-to-endosome cholesterol transport at membrane contact sites. *EMBO J*. 2017 May 15;36(10):1412-1433. doi: 10.15252/embj.201695917. Epub 2017 Apr 4.

Xu, J.M.; Wang, Y.; Wang, Y.L.; Wang, Y.; Liu, T.; Ni, M.; Li, M.S.; Lin, L.; Ge, F.J.; Gong, C.; et al. (2017) PIK3CA Mutations Contribute to Acquired Cetuximab Resistance in Patients with Metastatic Colorectal Cancer. *Clin. Cancer Res*. 2017, 23, 4602–4616.

Xu, Y.; Pasche, B. (2007) TGF-beta signaling alterations and susceptibility to colorectal cancer. *Hum. Mol. Genet.* 2007, *16*, 14–20.

Yarom, N., and Jonker, D.J. (2011). The Role of the Epidermal Growth Factor Receptor in the Mechanism and Treatment of Colorectal Cancer. *Discov. Med*. 11, 95–105.

Yau, T.O., Leung, T.H.Y., Lam, S., Cheung, O.F., Tung, E.K.K., Khong, P.L., Lam, A., Chung, S., and Ng, I.O.L. (2009). Deleted in liver cancer 2 (DLC2) was dispensable for development and its deficiency did not aggravate hepatocarcinogenesis. *PloS One* 4, e6566.

Yuan BZ, Miller MJ, Keck CL, Zimonjic DB, Thorgeirsson SS, Popescu NC. (1998) Cloning, characterization, and chromosomal localization of a gene frequently deleted in human liver cancer (DLC-1) homologous to rat RhoGAP. *Cancer Res*. 1998 May 15;58(10):2196-9.