NEW TARGET PROTEINS FOR DRUG DISCOVERY
IN COLON CANCER

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ABSTRACT

Background: Colorectal cancer (CRC) is one of the most common causes of death in Western countries. Because of the high heterogeneity and incidence of this disease, it is crucial to improve the knowledge on its biology – a fundamental step for drug discovery - and to develop clinically-relevant diagnostic and prognostic biomarkers.

Evidence suggests that MAGL [(a serine hydrolase that converts monoacylglycerols, such as the endocannabinoid 2-arachydonoyl glycerol (2-AG), in glycerol and fatty acid], NAAA [(a cysteine hydrolase responsible of the catabolism of palmitoylethanolamine (PEA)] and GPR35 (an orphan-G protein coupled receptor) affect biological events (e.g. proliferation, differentiation, survival) and regulates pathophysiological states (e.g. intestinal inflammation) which are suggestive of a possible involvement in CRC. Here, we explored the possible contribution of MAGL, NAAA and GPR35 to colon tumorigenesis.

Material and Methods: The role of MAGL, NAAA and GPR35 was assessed in vivo, via genetic or pharmacological blockade, in APC\textsuperscript{min} mice as well as in the azoxymethane (AOM), AOM/dextran sodium sulfate (DSS) and xenograft models.

Cell proliferation was evaluated in CRC cells, healthy colonic epithelial cells and colonic organoids 3D culture by using the BrdU and EdU incorporation; migration was examined in CRC and endothelial cells by using the scratch assay. Angiogenesis was assessed in tumour tissues [by microvessel counting and by investigating the expression of vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) proteins] as well as in the aortic ring model and in endothelial cells by using the tube formation assay. Cell metabolism was measured by the quantification of extracellular acidification rate (ECAR), oxygen consumption rate (OCR), lactate production and glucose uptake in bone marrow derived macrophages (BMDM). MAGL, NAAA and GPR35 expression was evaluated by RT-PCR and immunohistochemistry; 2-AG and PEA levels were measured by liquid chromatography mass spectrometry.

Results: MAGL- The MAGL inhibitor URB602 reduced xenograft tumour growth, the effect being associated to down-regulation of VEGF and FGF-2, reduction in the number of vessels and down-regulation of cyclin D1. A direct antiangiogenic effect was observed in human endothelial cells, too. In experiments aiming at investigating the role of MAGL in chemoprevention, URB602 attenuated AOM-induced preneoplastic lesions and tumours in wild-type but not in MAGL-deficient mice. NAAA- NAAA expression was reduced in biopsies of clinically-diagnosed CRC patients as well as in CRC cells incubated with tumour secretome. The NAAA inhibitor AM9053 and its substrate PEA inhibited proliferation and migration in CRC cells. Increased PEA levels in vivo (via NAAA inhibition or by its exogenous administration) resulted in chemopreventive effects
in the AOM model of colon carcinogenesis and reduced xenograft tumour growth. **GPR35**- GPR35 deletion resulted in a reduction of cell energetic demand and production in M0-, M1- and M2-BMDM. Also, depletion of GPR35 in M2-BMDM decreased their capability to produce the CXCL1 (pro-angiogenic chemokine) and to stimulate the tube formation of endothelial cells. Also, **GPR35**/− mice showed a reduced intestinal turnover in physiological conditions and, importantly, a reduced colon tumorigenesis, as highlighted in two different experimental models of colon cancer. It is noteworthy that the protective role of GPR35 in APC<sup>min</sup> mice was confirmed with the conditional deletion of *Gpr35* (Villin-Cre) in the intestinal epithelium.

**Conclusions:** In summary, by elucidating the physiopathological role of MAGL, NAAA and GPR35 in experimental colon tumorigenesis and by ascertain their dysregulation in intestinal tumours, this PhD thesis put forth such proteins as possible innovative prognostic markers in clinically-diagnosed CRC and as new molecular targets to be explored in drug discovery.
## ABBREVIATION LIST

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<tr>
<td>15-LOX</td>
<td>15-lipoxygenase</td>
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<td>2-AG</td>
<td>2-arachidonoyl glycerol</td>
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<td>2-deoxy-D-glucose</td>
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<td>Adenosine-A₃</td>
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<td>N-arachidonylethanolamide or anandamide</td>
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<td>AOM</td>
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<tr>
<td>ACF</td>
<td>adenomatous polyposis coli</td>
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<td>ATCC</td>
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<td>CXC-chemokine ligand</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
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<td>dextran sodium sulphate</td>
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<td>ECAR</td>
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<td>extracellular matrix</td>
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<td>EGF</td>
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<td>ELISA</td>
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<td>EMT</td>
<td>endocannabinoid membrane transporter</td>
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<td>EVs</td>
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<td>FAAH</td>
<td>fatty acid amide hydrolase</td>
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<td>FAP</td>
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<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FGF2</td>
<td>fibroblast growth factor 2</td>
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<tr>
<td>FITC</td>
<td>fluorescein 5-isothiocyanate</td>
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<td>FOLFIRI</td>
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<td>FOLFOX</td>
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<td>GPCR</td>
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<td>GWAS</td>
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<td>HCEC</td>
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<td>HCT116</td>
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<td>HUVEC</td>
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**CHEMICAL NAMES**

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<td>S-OOPP</td>
<td>N-[(3S)-2-oxooxetan-3-yl]-3-phenylpropanamide</td>
</tr>
<tr>
<td>URB602</td>
<td>cyclohexyl N-(3-phenylphenyl)carbamate</td>
</tr>
</tbody>
</table>
INTRODUCTION

Chapter I. Colorectal cancer (CRC)

1.1 Physiopathology and statistics

Colorectal cancer (CRC) represents a common cause of cancer-related deaths. It has been estimated that, in 2018, it will be the third most common cause of deaths in USA, with an equal incidence in males and females (8%) and with a prediction of 140,250 new diagnosed cases (Siegel et al., 2018; Figure 1). Similarly, CRC has been placed at the second position in the oncologic death rank in European countries for 2018 (Malvezzi et al., 2018; Figure 2) and in Italy 53,000 new CRC cases has been estimated to be diagnosed in the same year (www.airtum.it).

The majority of colorectal cancers are sporadic, arising from dysplastic adenomatous polyps. In this case, CRC develops as a consequence of the progressive accumulation of genetic and epigenetic alterations that cause transformation and progression of normal mucosa to adenoma and subsequently to carcinoma (Lao and Grady, 2011) (Figure 3). These alterations are the consequence of mutations in genes involved in cell growth regulation, such as tumour-suppressor genes (e.g. APC, Smad4 and p53) or oncogenes (e.g. K-Ras, c-myc, c-neu, c-src) (Calvert and Frucht H, 2002). This multi-step process spans 10 to 15 years, thereby providing an opportunity for prevention.

It is also well established that CRC is largely influenced by lifestyle, genetic predisposition and longstanding intestinal inflammation. About 10% of CRCs develops in the setting of well-defined hereditary syndromes i.e. hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) (Lynch and de la Chapelle, 2003; Rustgi, 2007).

Chronic gut inflammation is one of the main causes of increased risk of developing CRC (Terzic et al., 2010; Ullman and Itzkowitz, 2011) (Figure 3). Patients affected by inflammatory bowel diseases (IBD), such as ulcerative colitis (UC) and Crohn’s disease (CD), are at increased risk of developing neoplasia (Gupta et al., 2007; Itzkowitz and Harpaz, 2004), with an extended incidence rate of 2.75 and 2.64 of CRC in patients with UC and CD, respectively (Bernstein et al., 2001). It is worth noting that colitis can promote tumorigenesis by altering microbial composition as well as the expansion of microorganisms with genotoxic capabilities (Richard et al., 2018; Dejea et al., 2018). Other risk factors include obesity, meat and fat-rich diet, smoking, alcohol consumption and hyperinsulinemia (Marley and Nan, 2016; Wynder et al., 1969).

CRC is largely asymptomatic until the latter stages, when the cancer has already metastasized, thus a huge emphasis is also placed on early detection. Standard clinical practice for CRC patients include primarily the surgical resection, followed by neoadjuvant radiotherapy (for patients with rectal cancer), and adjuvant chemotherapy (for patients with stage III/IV and high-risk stage II colon cancer) (Marley and Nan, 2016). 5-fluorouracil (5-FU) is used in several poly-chemotherapy
regimens in CRC advanced patients, such as FOLFOX (Fluorouracil/Leucovorin and Oxaliplatin) and FOLFIRI [Fluorouracil/Leucovorin and Irinotecan (IR)] (Gustavsson et al., 2015). However, intrinsic or acquired resistance to 5-FU may occur (Ahn et al., 2015).

Despite the clear progresses raised in colon cancer diagnosis and therapy, the number of deaths still remain unacceptably high. Therefore, it is crucial to improve the knowledge on CRC pathogenesis, and, importantly, to identify new strategies, including chemoprevention and early diagnosis, in order to reduce the public health burden of this disease.

1.2 Microenvironmental regulation of tumour angiogenesis

Microenvironment plays a crucial role in the tumour growth and a considerable number of key transduction signals are involved in tumour microenvironment progression. Specifically, it is largely reported the importance of angiogenesis for the development of solid tumours, because the dissemination of tumours requires the growth of new blood vessels (Risau, 1997; Hanahan and Folkman, 1996).

Hypoxic cancer cells secrete vascular endothelial growth factor A (VEGFA), which initiates tumour angiogenesis by engaging VEGF receptor 2 expressed on the endothelial cells of neighbouring blood vessels (Potente et al., 2011). In pre-malignant stages of epithelial tumours, a basal lamina precludes the interaction between early lesions and the vascularized peritumoral tissues (Bossi et al., 1995; Bluff et al., 2009) (Figure 4). Instead, in the malignant tumours, cancer cells acquire invasive behaviours and induce a stromal response involving the formation of new blood vessels (Bergers and Benjamin, 2003) (Figure 4). Tumour-associated blood vessels typically acquire an aberrant morphology, which indicates the impaired vascular maturation, poor vessel functionality and incoherent tumour perfusion (Potente et al., 2011; Bergers and Benjamin, 2003; Morikawa et al., 2002). This angiogenic switch, strictly correlated with cancer progression, shows substantial variances in the patterns of tumour vascularization, which reflects differences in tumour type, grade and stage, anatomical site and expression of pro-angiogenic and anti-angiogenic factors (Bluff et al., 2009; Fukumura et al., 1997; Jubb et al., 2011; Morrissey et al., 2008).

Concerning CRC, it is characterised by enhanced VEGF expression and high microvascular densities, - suggestive of increased angiogenic activity - which negatively affect patient survival (Des Guetz et al., 2006). Consistently, therapy with anti-VEGF antibodies improves CRC patient survival, thus emphasising VEGF as a major clinical angiogenic factor (Carmeliet, 2005; Ferrara et al., 2007; Hawinkels et al., 2008).

Although cancer cells play a pivotal role as source of VEGFA and other pro-angiogenic mediators, many signals are produced from various tumour-associated stromal cells (TASCs), including bone marrow-recruited cells (e.g. macrophages, lymphocytes and neutrophils) as well as tissue-resident
cells such as vascular cells (i.e. endothelial cells and pericytes) and fibroblasts (Fang and Salven, 2011) (Figure 5).

In mouse models of cancer, macrophages derive mainly from circulating monocytes that extravasate into tumours in response to various chemoattractant stimuli (Qian and Pollard, 2010; Lahmar et al., 2016). Thereafter, monocytes differentiate and mature into tumour-associated macrophages (TAMs), i.e. the major constituents of malignant tumours - under the influence of colony-stimulating factor 1 (M-CSF) (Qian and Pollard, 2010; Lahmar et al., 2016).

Current therapeutic interventions often fail to prevent disease progression to metastatic dissemination. Hence, innovative therapeutic approaches include the targeting of specific molecular pathways such as proteins involved in the tumour angiogenesis and metastasis (Gelmon et al., 1999; Goetz et al., 2003).
### Estimated New Cases

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate</td>
<td>164,690</td>
<td>Breast</td>
<td>266,120</td>
<td>30%</td>
</tr>
<tr>
<td>Lung &amp; bronchus</td>
<td>121,680</td>
<td>Lung &amp; bronchus</td>
<td>112,350</td>
<td>13%</td>
</tr>
<tr>
<td>Colon &amp; rectum</td>
<td>75,610</td>
<td>Colon &amp; rectum</td>
<td>64,640</td>
<td>7%</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>62,380</td>
<td>Uterine corpus</td>
<td>63,230</td>
<td>7%</td>
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<tr>
<td>Melanoma of the skin</td>
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<td>Thyroid</td>
<td>40,900</td>
<td>5%</td>
</tr>
<tr>
<td>Kidney &amp; renal pelvis</td>
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<td>Melanoma of the skin</td>
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</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
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</tr>
<tr>
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<td>Pancreas</td>
<td>26,240</td>
<td>3%</td>
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<tr>
<td>Leukemia</td>
<td>35,030</td>
<td>Leukemia</td>
<td>25,270</td>
<td>3%</td>
</tr>
<tr>
<td>Liver &amp; intrahepatic bile duct</td>
<td>30,610</td>
<td>Kidney &amp; renal pelvis</td>
<td>22,660</td>
<td>3%</td>
</tr>
<tr>
<td><strong>All Sites</strong></td>
<td><strong>856,370</strong></td>
<td><strong>All Sites</strong></td>
<td><strong>878,980</strong></td>
<td><strong>100%</strong></td>
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</table>

### Estimated Deaths

<table>
<thead>
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<th>Females</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
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<td>Lung &amp; bronchus</td>
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<td>Pancreas</td>
<td>21,310</td>
<td>7%</td>
</tr>
<tr>
<td>Liver &amp; intrahepatic bile duct</td>
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<td>Ovary</td>
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</tr>
<tr>
<td>Leukemia</td>
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<td>Uterine corpus</td>
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<td>4%</td>
</tr>
<tr>
<td>Esophagus</td>
<td>12,850</td>
<td>Leukemia</td>
<td>10,100</td>
<td>4%</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>12,520</td>
<td>Liver &amp; intrahepatic bile duct</td>
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<td>3%</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
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<td>Non-Hodgkin lymphoma</td>
<td>8,400</td>
<td>3%</td>
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<td>Kidney &amp; renal pelvis</td>
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<td>Brain &amp; other nervous system</td>
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<td>3%</td>
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<tr>
<td><strong>All Sites</strong></td>
<td><strong>323,630</strong></td>
<td><strong>All Sites</strong></td>
<td><strong>266,010</strong></td>
<td><strong>100%</strong></td>
</tr>
</tbody>
</table>

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**Figure 1. Ten Leading Cancer Types for the Estimated New Cancer Cases and Deaths by Sex, United States, 2018** (from Siegel, 2018).

Estimates are rounded to the nearest 10 and cases exclude basal cell and squamous cell skin cancers and in situ carcinoma except urinary bladder. Ranking is based on modeled projections and may differ from the most recent observed data.
Figure 2. Prediction of cancer mortality in the European Union (EU) for the year 2018 (from Malvezzi et al., 2018).

Bar-plots of age-standardized (world population) death rates per 100 000 persons for the year 2012 (blue) and predicted rates for 2018 (green) with 95% prediction intervals for total cancer and the eight major cancer sites in EU men and women.
Canonical mechanisms of sporadic colorectal cancer (CRC) and colitis-associated cancer (CAC) development are shown in the top and bottom panels, respectively. CRC and CAC share similarities in their developmental pathways, including microsatellite instability (MSI), activation of the oncogene KRAS, activation of cyclooxygenase 2 (COX2; encoded by PTGS2), and mutation and eventual loss of heterozygosity (LOH) of TP53, adenomatous polyposis coli (APC), deleted in colon cancer (DCC) and SMAD4. However, the frequency and sequence of these events differs between the cancers. For example, mutation in APC is one of the first events in CRC, whereas it occurs at later stages in CAC. By contrast, TP53 mutations usually occur early in CAC but at a later stage in the progression of CRC. Although CRC shows a clear progression of morphological changes, from polyp to carcinoma, CAC progression involves increasing histological grades of dysplasia that culminate in an invasive carcinoma. CTNNB1, gene encoding β-catenin; CIN, chromosomal instability; IEC, intestinal epithelial cell.

Figure 3. Adenoma to carcinoma sequence (from West et al., 2015)
Introduction - Chapter I
Colorectal cancer

Figure 4. Angiogenesis during malignant progression (from De Palma et al., 2017)
Early-stage (pre-malignant) tumours typically display scant or no intratumoural vascularization, although a vascularized stroma surrounds the tumours and may adjoin parenchymal tumour domains (left panel). In malignant tumours, the cancer cells acquire invasive behaviours and induce a stromal response involving robust intratumoural angiogenesis, along with leukocyte infiltration, fibroblast proliferation and extracellular matrix (ECM) deposition (right panel). In pre-malignant lesions, a basal lamina separates the tumour from the surrounding tissues; this, together with angiostatic signals conveyed by some ECM components, and the relatively low levels of pro-angiogenic factors, prevents intratumoural vascularization or constrains it into a quiescent state. In malignant lesions, angiogenesis is largely controlled through the actions on vascular endothelial cells (ECs) of multiple pro-angiogenic mediators, which include growth factors, cytokines, various ECM proteins, ECM-remodelling enzymes, as well as extracellular vesicles (EVs) and by-products of deregulated tumour metabolism. ANGPT, angiopoietin; CXCL, CXC-chemokine ligand; EV, extracellular vesicle; FGF2, fibroblast growth factor 2; IFNa, interferon-α; IL, interleukin; MMP, matrix metalloproteinase; PAI1, plasminogen activator inhibitor 1; PDGF, platelet-derived growth factor; PIGF, placental growth factor; TABV, tumour-associated blood vessel; THBS1, thrombospondin 1; TNF, tumour necrosis factor; VEGFA, vascular endothelial growth factor A.
Figure 5. Tumour microenvironment and role of the inflammation (from West et al., 2015)

Production of inflammatory cytokines by several cell types including CD4+ T cells, innate lymphoid cells (ILCs) and tumour-associated macrophages (TAMs). Many of the inflammatory cytokines can act directly on transformed intestinal epithelial cells (IECs) to promote proliferation, inhibition of apoptosis, invasion, angiogenesis, epithelial to mesenchymal transition (EMT) and metastasis. More recently, it has been appreciated that these cytokines can activate cancer-associated fibroblasts (CAFs) to produce cytokines and growth factors that modulate both neoplastic cells and the tumour microenvironment.
Chapter II. Monoacylglycerol lipase (MAGL)

1.1 Monoacylglycerol lipase (MAGL): biochemistry and physiopathology

Monoacylglycerol lipase (MAGL) is a serine hydrolase that converts monoacylglycerols, such as the endocannabinoid 2-arachydonoyl glycerol (2-AG) to glycerol and fatty (arachidonic) acid. MAGL consists of two tissue specific isoforms with a molecular weights of 33 kD and 36 kD, which could reflect different splice variants (Karlsson et al., 2001; Long et al., 2009a). The enzyme associates with membranes and its active site resides within the cytosol. MAGL is localized in different areas of brain and in peripheral tissues, including the gastrointestinal tract (Ahn et al., 2008; Duncan et al., 2008; Izzo and Camilleri, 2009). MAGL blockade shows tissue-specific differences in monoacylglycerol metabolism, with the brain showing the most dramatic elevations in 2-AG and peripheral tissues often showing greater changes in other monoacylglycerols, consistent with the lipolytic role of MAGL as the final step of triglyceride hydrolysis in peripheral tissues (Long et al., 2009b).

In addition to the role of MAGL in terminating 2-AG signaling, MAGL exerts a crucial control over brain prostaglandins production in both basal and neuroinflammatory states. (Nomura et al., 2011a). In fact, through the hydrolysis of 2-AG, MAGL releases arachidonic acid (AA), i.e. the major precursor for the synthesis of pro-inflammatory eicosanoids (Nomura et al., 2011a). MAGL blockade decreases AA levels in the brain, stoichiometrically to 2-AG elevation, which also results in a reduction of lipopolysaccharide (LPS)-induced pro-inflammatory levels of downstream COX-driven prostaglandins [i.e. prostaglandin E2 (PGE2), PGD2, PGF2], and thromboxane production (Nomura et al., 2011a). For this reason, MAGL is an important target for neuroinflammatory and neurodegenerative disorders.

In recent years, the understanding of MAGL role has greatly increased, due to the progress made in the knowledge of the structure of MAGL, the synthesis of highly potent and selective in vivo efficacious inhibitors, e.g. JZL184 and URB02, as well as the development of MAGL-deficient mice (Chanda et al., 2010; Long et al., 2009a; Sclosburg et al., 2010) (Figure 6). Consequently, an impressive number of studies have described the therapeutic potential of MAGL inhibitors in a variety of human diseases – including cancer - through the bidirectional manipulation of endocannabinoids, eicosanoids, and other lipid signaling pathways.

1.2 2-arachydonoyl glycerol (2-AG) is a MAGL substrate

MAGL plays a predominant role in catalyzing the hydrolysis of the endocannabinoid 2-arachydonoyl glycerol (2-AG) (Dihn et al., 2002; Dihn et al., 2004; Hohmann et al., 2005). 2-AG is generated “on demand” through stimulus-dependent cleavage of membrane phospholipid precursors and its levels are regulated by the balance between its production and degradation (Murataeva et al.,
2014). 2-AG is thought to be produced through hydrolysis of phospholipids by phospholipase C (PLC) β or δ to release diacylglycerols (DAG), which are then degraded to 2-AG by diacylglycerol lipase (DAGL) α or β (Gao et al., 2010; Tanimura et al., 2010). (Figure 7).

Genetic or pharmacological inhibition of MAGL reduces 2-AG hydrolysis by >80% in most tissues, including the brain. The remaining amount of 2-AG is metabolized by the hydrolytic enzymes α/β-hydrolase domain 6 (ABHD6), ABDH12, fatty acid amide hydrolase (FAAH) (Blankman et al., 2007; Dihn et al., 2004; Marrs et al., 2010; Goparaju et al., 1998), 15-lipoxygenase (Kozak et al., 2002) and monoacylglycerol acyltransferases/kinases (Simpson et al., 1991). Although the other enzymes may have roles in 2-AG hydrolysis in certain settings, MAGL blockade lead to dramatic elevations, especially in the brain, in 2-AG levels, confirming that MAGL is the primary enzyme involved in degrading 2-AG in vivo (Long et al., 2009b; Nomura et al., 2011a; Schlosburg et al., 2010; Fowler, 2012).

2-AG is an endogenous lipid mediator involved in a variety of physiological and physiopathological processes. 2-AG behaves as full agonist for the cannabinoid (CB)1 and CB2 receptors (Sugiura et al., 1999; Sugiura et al., 2000). The 2-AG homologues 2-linoleoylglycerol and 2-palmitoylglycerol, which are present in tissues and presumably released together with 2-AG, act as ‘entourage compounds’ to potentiate the effects of 2-AG at CB receptors without themselves having a direct action on CB receptors (Ben-Shabat et al., 1998). In addition, several studies report that 2-AG may also exert physiological effects independently from CB1 and CB2 receptors activation, for example by binding to and activating GABA<sub>A</sub> (Sigel et al., 2011), PPARγ (Bouaboula et al., 2005), adenosine A3 (Lane et al., 2010), TRPV1 (Iwasaki et al., 2008), and GPR55 (Ryberg et al., 2007) (Figure 7). However, CB1 receptor is the major brain target through which 2-AG exerts its physiopathological effects, including regulation of food intake and metabolism, neuro-inflammation, addiction, anxiety and pain (Baggelaar et al., 2018).

1.3 The role of MAGL and its substrate 2-AG in cancer

MAGL is up-regulated in aggressive human ovarian, prostate, breast cancer cells and in primary tumours, where it promotes migration, invasion, survival, and in vivo tumour growth (Mulvihill and Nomura, 2013; Nomura et al., 2010; Nomura et al., 2011b). In cancer cells, MAGL plays a role in controlling global levels of free fatty acids, which serve as building blocks for the synthesis of pro-tumorigenic signalling lipids, such as prostaglandin E2 and lysophosphatidic acid (Qin et al., 2014; Nomura et al., 2010). Recently, it has been reported that MAGL is upregulated in melanoma tissue and its expression in tumour cells is significantly associated with tumour aggressiveness, vascular invasion of the primary lesion and tumour progression (Baba et al., 2017).
Concerning 2-AG, i.e. the main substrate of MAGL, studies have shown that it exerts antiproliferative effects in a number of cancer cell lines (Melck et al., 2000; Costa et al., 2014; Orellana-Serradell et al., 2015), including colorectal cancer cells (Ligresti et al., 2003). For example, the increase of endogenous 2-AG levels as a consequence of MAGL inhibition was shown to reduce prostate cancer invasion \textit{in vitro} (Nomura et al., 2011b). On the other hand, the reduction of physiological endogenous 2-AG levels has been reported to stimulate cell invasion (Nithipatikom et al., 2011).

Our understanding of how MAGL impacts colon cancer progression is so far hindered by under-reported and controversial data (Schicho and Storr, 2011; Izzo et al., 2015), since the enzyme has been associated to both cancerogenic (Sun et al., 2013) and anti-cancerogenic processes (Ye et al., 2011). Nevertheless, the effect and role of MAGL in relation to 2-AG production and angiogenesis have been not evaluated to date.
Figure 6. Conversion of 2-Arachidonoylglycerol (2-AG) into arachidonic acid and glycerol by monoacylglycerol lipase (MAGL). MAGL inhibitors such as URB602 and JZL184 increase the endogenous levels of 2-AG.
Figure 7. Metabolic pathways and molecular targets of 2-AG (adapted from Iannotti and Piscitelli, 2018).

ABH4/6/12, αβ-hydrolase 4/6/12; CB1/2, cannabinoid receptor 1/2; COX2, cyclooxygenase 2; DAG, diacylglycerol; EMT, 'endocannabinoid membrane transporter'; FAAH, fatty acid amide hydrolase; GDE1, glycerophosphodiester phosphodiesterase; GPR55, G-protein-coupled receptor 55; PLCβ, phospholipase Cβ; PLD, phospholipase D; PTPN22, protein tyrosine phosphatase, nonreceptor type 22; TRPV1, transient receptor potential, vanilloid subtype 1 receptor; GABAA, type-A γ-aminobutyric acid receptor; adenosine A3, adenosine A3 receptor; PPARγ, peroxisome proliferator-activated receptor gamma; 15-LOX, lipoxygenase-15; PMs, prostaglandin-ethanolamides/prostamides; PG-GEs, prostaglandin-glyceryl esters.
Chapter III. N-acylethanolamine acid amide hydrolase (NAAA)

1.1. N-acylethanolamine acid amide hydrolase (NAAA): biochemistry and physiopathology

N-acylethanolamine acid amide hydrolase (NAAA) is a N-terminal cysteine hydrolase of the choloylglycine hydrolase family. Being a lysosomal enzyme, NAAA is produced as an inactive pro-enzyme and autocatalytically activated by cleavage at acidic pH. Human and murine NAAA are similar with 76% sequence identity (Tsuboi et al. 2005). Glycosylation of the enzyme was firstly believed to be necessary to reach its full activity. However, more recent findings have demonstrated that glycosylation is most probably necessary for trafficking of the enzyme to the lysosomes and its maturation, with minimal effects on enzyme activity (Pavlopoulos et al. 2018).

NAAA is responsible of the hydrolysis of N-acylethanolamines (NAEs), mainly PEA and to a very less extent OEA and AEA. Consistently, NAAA inhibitors increase preferentially PEA levels in vitro and in vivo, making NAAA a promising target for drug discovery (Tsuboi et al. 2005; Ueda et al., 2013; Alhouayek and Muccioli, 2014; Bottemanne et al., 2018) (Figure 8). With the recent molecular identification of NAAA, a number of selective and stable NAAA inhibitors have been synthesised and tested in a variety of disease models. While the first generation of inhibitors were irreversible and moderately potent, in more recent years new potent, reversible, stable and selective inhibitors have been synthesised (Bottemanne et al., 2018; Figure 8). Makriyannis’s research group developed a series of isothiocyanate-based NAAA reversible inhibitors such as AM9053 and AM9023 (West et al., 2012; Malamas et al., 2015) (Table 1). Other series of potent NAAA inhibitors were developed by Piomelli and co-workers around β-lactone and β-lactam moieties, such as β-lactone (S)-OOPP, ARN077 and ARN726 (Solorzano et al., 2009; Duranti et al., 2012; Ponzano et al., 2013; Ribeiro et al., 2015; Nuzzi et al., 2016) (Table 1). These new inhibitors are precious tools to study the role of NAAA.

Because NAAA is highly expressed in bone marrow and immune system, especially in macrophages, its pathophysiological role was firstly evaluated in inflammatory processes (Solorzano et al., 2009; Sasso et al., 2013; Yang et al., 2015). Moreover, because NAAA is believed to primarily control PEA levels (Tsuboi et al. 2005; Tai et al., 2012), its inhibition is expected to mimic the anti-inflammatory, neuroprotective and analgesic properties of PEA (Alhouayek and Muccioli, 2014). NAAA is also largely expressed in human and murine small and large intestine (Borrelli et al., 2015; Alhouayek et al., 2015) and its pharmacological inhibition reduces colon inflammation by locally increase PEA levels (Alhouayek et al., 2015). However, it is important to note that NAE levels as well as the expression of NAAA may be altered differently depending on the tissue or the disease considered.
1.2 Palmitoylethanolamide (PEA) is a NAAA substrate

N-acylethanolamines (NAEs) are endogenous lipids consisting of an acyl chain linked by an amide bond to ethanolamine. Although sharing the same basic scaffold, NAEs can bind to several different receptors and exert a plethora of biological effects (Bottemanne et al., 2018). Besides the well characterized NAEs, such as the endocannabinoid N-arachidonoylethanolamine (AEA), N-palmitoylethanolamine (PEA) is one of the most-studied NAEs, being it clinically used for its anti-inflammatory, analgesic and neuroprotective properties mostly mediated by PPAR-α (Alhouayek and Muccioli, 2014; Gugliandolo et al., 2018; Esposito and Cuzzocrea, 2013; Skaper et al., 2015; Iannotti et al., 2016). Despite PPARα is the main reported target (Lo Verme et al., 2005), additional and pharmacologically relevant targets of PEA have been reported (Petrosino and Di Marzo, 2017) (Figure 9). Thus, PEA has also been shown to be an agonist of orphan G-protein coupled receptor 55 (GPR55) (Ryberg et al., 2007), although this pharmacological implication remains to be clarified. In addition, unlike AEA, PEA does not bind to cannabinoid (CB) receptors, although it can indirectly activate them via the so-called “entourage” effect, i.e. through the inhibition of FAAH, the enzyme responsible for endocannabinoids degradation (Di Marzo et al., 2001; Petrosino et al., 2016). Finally, PEA may directly or indirectly modulate (via enhancement of AEA levels or via PPAR-α activation) transient receptor potential cation channel subfamily V (TRPV)1 channels (De Petrocellis et al., 2002; Ho et al., 2008; Ambrosino et al., 2013; Ambrosino et al., 2014) (Figure 9).

In addition to be an endogenous lipid molecule, PEA is also a food component, first discovered in the late 1950s, when it was shown that the anti-allergic and anti-inflammatory activities exerted by peanut oil or soybean lecithin were due to a specific lipid fraction corresponding to PEA (Coburn et al., 1954; Ganley et al., 1958). PEA has been detected in a wide variety of food sources such as milk, tomato and corn (Venables et al., 2005; Kilaru et al., 2007; Gouveia-Figueira and Nording, 2014) (Table 2).

1.3 The role of NAAA and its substrate PEA in cancer

Several splice variants of NAAA are expressed in human cells. Among these, two code for catalytically inactive proteins which represent up to 20% of the expressed NAAA in certain cell lines, including the human prostate cancer cell line VCAP) (Sakura et al., 2016). The expression and the activity of NAAA were reported in a number of tumour types and the enzyme has been also proposed as a possible biomarker of prostate cancer (Wang et al., 2008). More recently, NAAA expression was found higher in non-aggressive prostate cancer, assuming the enzyme as a promising signature for tumour aggressiveness (Liu et al., 2014). Also, NAAA is highly expressed in aggressive mouse ovarian cancer (Du et al., 2016). Interestingly, it has been recently reported
that pharmacological inhibition of NAAA decreased proliferation and migration and caused cell death in different bladder cancer cell lines (Vago et al., 2017). However, to date there is no information about the possible role of NAAA in colorectal cancer physiopathology.

Concerning PEA, there are some findings which are suggestive of a possible role in cancer. First, some of PEA targets (e.g. CB receptors, TRPV1 and PPAR-α) are involved in the carcinogenesis mechanisms. Furthermore, PEA has been shown: i) to slow up melanoma cell survival (Hamtiaux et al., 2012); ii) to induce cell death in high grade astrocytomamas/neuroblastoma cells (Stock et al., 2012); iii) to be dramatically decreased in human tumour brain tissues compared to healthy area (Maccarone et al., 2001) and iv) to potentiate the cytotoxic effect of anandamide in human breast cancer cells (De Petrocellis et al., 2002). A recent study on colorectal cancer cells has shown antiproliferative and antiangiogenetic effects of PEA, possibly through a selective PPAR−α dependent inhibition of AKT/mTOR pathway (Sarnelli et al., 2016). Nevertheless, the knowledge of PEA role in CRC is still greatly fragmentary, lacking functional studies that corroborate its involvement and potential molecular mechanisms.
Figure 8. Conversion of palmitoylethanolamide (PEA) into palmitic acid and ethanolamide by acylethanolamine acid amide hydrolase (NAAA) (adapted from Bandiera et al., 2014). Representative chemical structures of NAAA inhibitors are shown. Specific NAAA inhibitors and their pharmacological properties are reported in Table 1.
### TABLE 1 Characteristics of representative NAAA inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>NAAA inhibition</th>
<th>FAAH inhibition</th>
<th>Effect on NAE levels</th>
<th>In vivo</th>
<th>Dose</th>
<th>Effect on NAE levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclopentyl hexadecanoate</td>
<td>IC\textsubscript{50} = 10 µM (hNAAA)</td>
<td>FAAH: no significant effect (at 50 µM)</td>
<td>Increases PEA levels in NAAA-expressing HEK cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrrolidine derivative 16</td>
<td>IC\textsubscript{50} = 2 µM (rNAAA)</td>
<td>FAAH: IC\textsubscript{50} &gt;100 µM</td>
<td>Increases PEA levels in RAW264.7 cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM9023</td>
<td>IC\textsubscript{50} = 0.600 µM (hNAAA)</td>
<td>FAAH: IC\textsubscript{50} &gt;10 µM</td>
<td>Increases NAE levels in J774 cells</td>
<td>DSS-induced colitis</td>
<td>10 mg/kg, b.i.d. (i.p.)</td>
<td>Increases PEA levels in the colon</td>
</tr>
<tr>
<td>AM9053</td>
<td>K\textsubscript{i} = 0.030 µM (rNAAA)</td>
<td>FAAH: IC\textsubscript{50} = 100 µM</td>
<td>Increases NAE levels in RAW264.7 cells and in NAAA-expressing HEK cells (at 10 µM)</td>
<td>Carrageenan-instilled sponges to recruit leukocytes</td>
<td>25 µg/implanted sponge</td>
<td>Increases PEA levels in carrageenan-recruited leukocytes</td>
</tr>
<tr>
<td>(S)-OOPP</td>
<td>IC\textsubscript{50} = 0.420 µM (rNAAA)</td>
<td>FAAH: IC\textsubscript{50} = 100 µM</td>
<td>Increases PEA levels in RAW264.7 cells and in NAAA-expressing HEK cells (at 10 µM)</td>
<td>Carrageenan-induced lung inflammation</td>
<td>5–50 µg/paw (intraplantar)</td>
<td>Not reported</td>
</tr>
<tr>
<td>ARN077</td>
<td>IC\textsubscript{50} = 0.01 – 0.130 µM (rNAAA)</td>
<td>FAAH: no significant effect (at 10 µM)</td>
<td>Increases PEA levels in the sciatic nerve</td>
<td>Chronic constriction injury</td>
<td>20 µl of a 10% suspension topically administered on the paw skin</td>
<td>Increases PEA levels in the sciatic nerve</td>
</tr>
<tr>
<td>ARN726</td>
<td>IC\textsubscript{50} = 0.063 µM (rNAAA)</td>
<td>FAAH: IC\textsubscript{50} &gt;100 µM</td>
<td>Increased PEA and OEA levels in TPA-treated ears</td>
<td>Carrageenan-induced lung inflammation</td>
<td>30 mg/kg (per os)</td>
<td>Increased PEA and OEA levels</td>
</tr>
<tr>
<td>N-0-aryl-substituted derivative 37</td>
<td>IC\textsubscript{50} = 0.006 µM (hNAAA)</td>
<td>FAAH: IC\textsubscript{50} &gt;50 µM</td>
<td>Carrageenan-induced paw inflammation</td>
<td>CFA-injected paw</td>
<td>30 mg/kg (i.p.)</td>
<td>Increased PEA and OEA levels</td>
</tr>
<tr>
<td>F96</td>
<td>IC\textsubscript{50} = 0.270 µM (rNAAA)</td>
<td>FAAH: IC\textsubscript{50} = 41 µM</td>
<td>Increased PEA and OEA levels in TPA-treated ears</td>
<td>TPA-induced ear edema</td>
<td>10 mg/kg (i.p.)</td>
<td>Increased PEA and OEA levels</td>
</tr>
<tr>
<td>F215</td>
<td>IC\textsubscript{50} = 0.009 µM (rNAAA)</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(from Bottemanne et al., 2018)
Figure 9. Metabolic pathways and molecular targets of PEA (from Petrosino and Di Marzo, 2017).

(A) PEA is biosynthesized from a membrane phospholipid, N-palmitoylphosphatidylethanolamine (NPPE), via several routes, the most investigated of which is through the direct hydrolysis by NAPE-PLD. PEA can be then degraded to palmitic acid and ethanolamine by either FAAH or NAAA (Iannotti et al., 2016). NAT, N-acyl-transferase.

(B) PEA can directly activate PPAR-α (Lo Verme et al., 2005b) or, more controversially, GPR55 (Ryberg et al., 2007).

(C) PEA, for example through the inhibition of the expression of FAAH, may increase the endogenous levels of AEA and 2-AG, which directly activate CB2 (or CB1) receptors and TRPV1 channels (entourage effect) (Di Marzo et al., 2001; Petrosino et al., 2016).

(D) PEA, possibly through an allosteric modulation of TRPV1 channels, potentiates the activation and desensitization by AEA and 2-AG of TRPV1 channels (entourage effect) (De Petrocellis et al., 2001; Di Marzo et al., 2001; Ho et al., 2008; Petrosino et al., 2016).

(E) PEA may also activate TRPV1 channels via PPAR-α (Ambrosino et al., 2013, Ambrosino et al., 2014).
TABLE 2. Food sources that contain PEA

<table>
<thead>
<tr>
<th>Food source</th>
<th>Concentration of PEA (ng g⁻¹ fresh weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine milk</td>
<td>0.25</td>
</tr>
<tr>
<td>Elk milk</td>
<td>1.81</td>
</tr>
<tr>
<td>Human breast milk</td>
<td>8.98 ± 3.35 nmol·L⁻¹</td>
</tr>
<tr>
<td>Human breast milk (110 ± 32.3 lactation days)</td>
<td>23.4 ± 7.2 nmol·L⁻¹</td>
</tr>
<tr>
<td>Common bean (Phaseolus vulgaris)</td>
<td>53.5</td>
</tr>
<tr>
<td>Garden pea (Pisum sativum)</td>
<td>100</td>
</tr>
<tr>
<td>Southern or blackeyed peas (Vigna unguiculata)</td>
<td>138</td>
</tr>
<tr>
<td>Tomato</td>
<td>100</td>
</tr>
<tr>
<td>Medicago sativa</td>
<td>1150</td>
</tr>
<tr>
<td>Corn</td>
<td>200</td>
</tr>
<tr>
<td>Soybean (Glycine max)</td>
<td>6700</td>
</tr>
<tr>
<td>Soy lecithin</td>
<td>950000</td>
</tr>
<tr>
<td>Peanut (Arachis hypogaea)</td>
<td>3730</td>
</tr>
</tbody>
</table>

(from Petrosino and Di Marzo, 2017)
Chapter IV. G protein-coupled receptor 35 (GPR35)

1.1 Orphan G protein-coupled receptor 35 (GPR35)

The G protein-coupled receptors (GPCRs) superfamily of transmembrane-spanning proteins is composed of ~1,000 members (Lagerstrom and Schioth, 2008) and comprises ~3% of the human genome (Insel et al., 2007). Considering their ubiquity and central role in signal transduction, it is not surprising that GPCRs constitute the largest class of drug targets for a wide range of pathological conditions (Santos et al., 2017; Campbell and Smrcka, 2018). Although GPCRs-targeted therapies are hampered by undesirable side effects [due to a lack of receptor subtype selectivity (Wang and Lewis, 2013) and/or a pathological interference with physiological signaling (Kenakin, 2005)], GPCRs remain attractive targets for drug design. In last years, more than 140 GPCRs with unknown endogenous ligands have been discovered, including the so-called orphan receptors (Levoye et al., 2006). These are involved in different physiological effects and may provide access to signal transduction pathways currently unknown, allowing for new strategies in drug design. Importantly, orphan GPCRs-targeted therapies may be more selective than those currently known, resulting in a potential side effects reduction.

G protein-coupled receptor 35 (GPR35) is an orphan receptor discovered in 1988 (O’Dowd et al., 1998) (Figure 10). Despite being able to be activated by high concentration of endogenous molecules, foremost the tryptophan metabolite kynurenic acid (KYNA), 2-oleoyl lysophosphatidic acid (LPA), CXCL17 and cGMP (cyclic guanosine 3’5’ monophosphate), it has not been demonstrated that any of these agonists binds selectively to GPR35 (Oka et al. 2010; Wang et al. 2006; Maravillas-Montenero et al., 2015). For example, KYNA shows huge species-dependent differences in potency, which is lowest (EC_{50}>10^{-3} M) for human GPR35 (Jenkins et al., 2011; Milligan, 2011), and CXCL17 does not act as GPR35 agonist in some experimental system (Park et al., 2018).

A number of synthetic surrogate ligands for GPR35 have been proposed. One of the earliest and most useful GPR35 ligands is the cGMP phosphodiesterase inhibitor zaprinast (2-(2-propyloxyphenyl)-8-azapurin-6-one) (Taniguchi et al., 2006). Despite its wide application as reference GPR35 agonist (with reported potency between 2-8 μM at the human ortholog), zaprinast, similarly to kynurenic acid and many other synthetic agonists, display a marked animal (human vs rodent) species selectivity (Mackenzie et al., 2014; Jenkins et al. 2010, Jenkins et al. 2012).

GPR35 is mainly expressed in colon, spleen and immune cells (e.g. macrophages and dendritic cells) in both humans and mice (Maravillas-Monterro et al. 2015; Divorty et al. 2015; Taniguchi et al., 2006; Wang et al., 2006. In the rat, high expression of GPR35 has been found in spleen, colon, dorsal root ganglion, and uterus (Taniguchi et al., 2006; Ohshiro et al., 2008).
It has been reported that GPR35 plays a role in a wide range of human diseases, but the signaling pathways have been not elucidated (Sun 2008, Okumura et al., 2004). In terms of classical GPCR signaling, GPR35 appears to couple to various Gα subunits depending on the cell type and/or background animal species (Divorty et al. 2015).

1.2 GPR35: evidence supporting its involvement in cancer
A genome-wide association study (GWAS) identified a single-nucleotide polymorphism (SNP) of GPR35 as novel risk locus for ulcerative colitis (UC) and primary sclerosing cholangitis (PSC) (Ellinghaus et al. 2013). The GPR35 rs3749171 SNP, leading to a threonine to methionine shift at Thr3.44, is located in the third transmembrane helix of the receptor (Ellinghaus et al. 2013). The third intracellular domain with its DRY motif is crucial for keeping the receptor in its inactive state. Only after ligand binding GPCRs change their structure so that the DRY motif allows the activation of downstream signaling. Remarkably, patients with UC and PSC have a higher risk to develop cancer (Krugliak Cleveland et al. 2017, Karlsen et al. 2017, Ullman and Itzkowitz, 2011).

GPR35 is highly expressed in human gastric cancer cells (Okumura et al., 2004). Moreover, GPR35 was identified as a potential receptor for CXCL17, a mucosal chemokine that promotes tumour growth and angiogenesis (Maravillas-Montero et al. 2015, Weinstein et al. 2006). Thus, CXCL17 levels are significantly increased in primary colonic cancers and clinical analyses suggest that CXCL17 could be an important biomarker of CRC poor prognosis (Ohlsson et al. 2016). Recently, it has been proposed a clinical significance of the CXCL17-CXCR8 (GPR35) axis in breast cancer, but the mechanistic basis of this effect remains poorly understood (Guo et al., 2017).

Recently, a significant increase of GPR35 expression, correlating with poor prognosis, has been reported in non-small-cell lung cancer (NSCLC) tissues (Wang et al., 2018). Notably, GPR35 was upregulated in chemoresistance cell model of NSCLC, and GPR35-mediated chemoresistance occurred partially via β-arrestin-2/Akt signalling (Wang et al., 2018).

Despite these findings, GPR35 function as tumour suppressor or inducer in cancers is still uncertain and may be related to cancer/cell types and/or specific microenvironment of the tumour.

1.3 Glucose metabolism in cancer
Cancer cells metabolize glucose in a different manner compared to normal cells (Warburg, 1956). Glucose metabolism and glycolysis is accelerated in cancer cells by preferential expression of transporters and enzyme isoforms that drive glucose flux. While in normoxia, healthy cells use the degradation of glucose to pyruvate and later the TCA (tricarboxylic acid) cycle to produce ATP, neoplastic cells prefer to use glycolysis to produce energy rather than oxidative phosphorylation (Warburg, 1956) (Figure 11). Indeed, tumour cells switch metabolism with high lactate production, even in aerobic conditions, and mitochondrial metabolism suppression. This metabolic adaptation is
called “aerobic glycolysis” or the “Warburg effect” (Warburg, 1956) (Figure 11). In solid tumours, the hypoxic environment supports glycolytic metabolism and provides resistance to therapy as well as an optimal niche for the maintenance of cancer stem cells (Trédan et al., 2007; Persano et al., 2011; Fidoamore et al., 2016). Several genes (e.g. oncogenes) involved in the glycolytic pathway regulate the adjustment of cancer cells to the metabolic switch, suggesting them as possible target in cancer therapy.

Activation of macrophages or dendritic cells with a range of stimuli, including LPS (Krawczyk et al., 2010), the TLR3 ligand poly(I:C) (Pantel et al., 2014) and type I interferon (IFN) (Pantel et al., 2014), induces a metabolic switch fromOXPHOS to glycolysis, in a phenomenon similar to the Warburg effect (Pantel et al., 2014; Kelly and O’Neill, 2015) (Figure 12). Recent immunometabolism studies have shown that alterations in the metabolic profile of macrophages shape their activation state and function (Kelly and O’Neill, 2015). Thus, metabolic reprogramming of macrophages could become a therapeutic approach to treat diseases, such as cancer, with a high macrophage involvement (Geeraerts et al., 2017).

Here, we took advantage of macrophages in order to investigate the metabolic role of GPR35 and the possible impact on experimental models of colorectal cancer.
Figure 10. Helix net representation of human GPR35 receptor structure (from Shore and Reggio, 2015) and simplified activation of GPR35 by endogenous or synthetic ligands.

The most highly conserved residue in each transmembrane helix (among Class A GPCRs) is shown in red. Possible disulfide bridges are indicated by double-headed arrows. Tryptophan metabolite kynurenic acid (KYNA), 2-oleoyl lysophosphatidic acid (LPA),...
(A) In resting cells, glucose is metabolized to pyruvate via glycolysis. Some pyruvate is converted to lactate, but most is directed to the TCA cycle via acetyl-CoA. The TCA cycle generates NADH, which donates electrons to the mitochondrial electron transport chain so that OXPHOS can progress.

(B) In highly proliferative or tumour cells, the metabolic profile switches from OXPHOS to aerobic glycolysis, known as the Warburg effect. Mature innate immune cells also rely on glycolysis, although they do not proliferate after activation. The majority of the pyruvate generated by glycolysis is converted to lactate, and glycolytic intermediates build up, meeting the high energy demand of the cell. Glycolysis is the source of ATP in these cells, and also provides glucose-6-phosphate for nucleotide biosynthesis in the PPP.

Figure 11. The Warburg effect (from Kelly and O’Neill, 2015)
Figure 12. Physiological and metabolic differences between M1 and M2 phenotypes of macrophages (Adapted from Kelly and O’Neill, 2015)

M1 macrophages (pro-inflammatory phenotype) rely on glycolysis for ATP production and have increased levels of iNOS, HIF-1α and u-PFK2, while M2 macrophages (pro-resolving phenotype) are fueled by OXPHOS and have increased levels of Arg-1, AMPK and PFKFB1. M1 macrophages release pro-inflammatory cytokines such as IL-1β, while M2 macrophages are involved in the response to parasite infection, as well as in wound healing, and they release the anti-inflammatory cytokine IL-10. In fact, it is thought that a spectrum of macrophage activation exists, with different populations of macrophages exhibiting different inflammatory and metabolic phenotypes.
AIM

The general aim of this work is to investigate the possible role of three specific targets, namely two enzymes [i.e. monoacylglycerol lipase (MAGL) and N-acylethanolamine acid amide hydrolase (NAAA)] and the G protein-coupled receptor (GPR) 35 in colorectal cancer (CRC).

To adequately pursue our objective, both pharmacological and genetic blockade of MAGL, NAAA and GPR35 were exploited in well-established models of sporadic colon cancer (i.e. AOM-induced preneoplastic lesions, polyps and tumours and APC$^{min}$ mice), in a model of colitis associated to cancer (tumours induced by the AOM/DSS combination) and in xenograft CRC tumours.

More in depth studies were performed in colorectal cancer cell lines (in order to evaluate proliferation and migration), in endothelial cells (to evaluate the angiogenic process) and in bone marrow-derived macrophages (to evaluate the impact on metabolic profile).

Finally, we have measured the expression of these targets in human colonic biopsies of CRC patients, which is of obvious translational value.

In few words, the ultimate goal of my PhD thesis is to provide potential innovative therapeutic targets for CRC.
MATERIALS AND METHODS

1.1 Patients
Human studies were approved by Ethical Committee of the University of Naples Federico II, Department of Medicine (protocol number 245_2015). Patients with a well-established diagnosis of colorectal cancer (CRC, stage II-IV) were included. Human colon samples were obtained by surgical colorectal resection. Healthy tissues, obtained from the peritumoral or distal resection margins of tumour, were used as control. All patients provided written informed consent.

1.2 Mice
All procedures performed in studies involving animals were carried out in accordance with the Italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC and 2010/63/UE). Experiments were conducted in UK with the approval of the UK home office and in Italy with the approval of the Institutional Animal Ethics Committee for the use of experimental animals. Male ICR mice (weighting 25–30 g) and athymic nude female (4-weeks old) mice were purchased from Harlan Italy (S. Pietro al Natisone, UD, Italy) and fed ad libitum with standard food (Mucedola srl, Settimo Milanese, Italy). MAGL knock-out (MgL−/−) mice were a kind gift of R. Zechner and R. Zimmermann, University of Graz, Austria. GPR35 knock-out (Gpr35−/−) mice were obtained from the KOMP repository and the intestinal epithelia-specific GPR35 were generated by outbreeding the FlpO and then crossing into mice carrying Villin-Cre. Ear-biopsy genomic DNA was used for routine genotyping of all mice. GPR35 mice were bred and maintained in specific pathogen-free conditions at the Central Biomedical Services (CBS) facility, University of Cambridge. Athymic female mice, fed ad libitum with sterile mouse food, were maintained under pathogen-free conditions at the Department of Pharmacy, University of Naples Federico II. All mice were used after 1 week-acclimation period (temperature 23±2 °C; humidity 60%, free access to water and food).

1.3 Drugs
Azoxymethane (AOM) was purchased from Sigma (Milan, Italy). URB602 and MatrigelTM were obtained from Cayman Chemical (Cabru SAS, Arcore, Italy) and BD Biosciences (Buccinasco, Milan, Italy), respectively. DSS was obtained from MP Biomedicals (Canada). PEA was a kind gift from Epitech Group. AM9053 was synthesized in the laboratory of A. Makriyannis and M. Malamas. All reagents for cell cultures were obtained from Sigma, Bio-Rad Laboratories (Milan, Italy) and Microtech Srl (Naples, Italy). The vehicles used for in vivo (10% ethanol, 10% Tween-
Materials and Methods

20, 80% saline, 2 ml/kg) and in vitro (0.1% DMSO or 0.1% ethanol) experiments had no effect on the responses under study.

1.4 Cell culture

1.4.1 Colorectal cancer cell lines

For in vitro experiments, two human colon adenocarcinoma cell lines (i.e. Caco-2 and HCT116, ATCC from LGC Standards, Milan, Italy), with a different genetic profile (APC gene and p53 mutated in Caco-2 cells, K-RAS mutated in HCT116 cells) have been used (Ahmed et al. 2013). These cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, 1% non-essential amino acids, 2 mM L-glutamine and 1 M HEPES, in conformity with the manufacturer’s protocols. Cell viability was evaluated by trypan blue exclusion.

1.4.2 Healthy colonic epithelial cells (HCEC)

The immortalized healthy human colonic epithelial cells (HCEC), derived from human colon biopsies, have been used as a comparison with tumoural cells. HCEC were a kind gift of Fondazione Callerio Onlus (Trieste, Italy). HCEC were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 100 Units/ml penicillin, 100 μg/ml streptomycin, 200 mM L-Glutamine, 100 mM Na-pyruvate and 1 M HEPES. Cell viability was evaluated by trypan blue exclusion.

1.4.3 Endothelial cell lines

The human umbilical vein endothelial cell line (HUVEC, Promocell, Heidelberg, Germany) and immortalized murine endothelial cells (SVEC4-10, ATCC from LGC Standards, UK) were used. Cells were routinely maintained in 75 cm² polystyrene flasks, at 37 °C in a 5% CO₂ atmosphere, in Dulbecco’s modified Eagle’s medium (DMEM) for SVEC4-10 or in endothelial growth medium (EGM-2) for HUVEC. DMEM was supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 1% non-essential amino acids and 2 mM L-glutamine. EGM-2, containing VEGF, R3-IGF-1, hEGF, hFGF, hydrocortisone, ascorbic acid, heparin and GA-1000 (Clonetics, Cambrex Bio Science Walkersville, USA) was supplemented with 10% FBS.

1.5 Primary cells isolation and culture

1.5.1 Murine bone marrow-derived macrophages (BMDM)

Bone marrow was flushed out of the murine femur and tibia with macrophages culture medium (RPMI 1640, supplemented with 1 mM HEPES pH 7.4, 50 U/ml PenStrep and 10 % FBS). Cells were then filtered through 70 μm cell strainer and incubated for 6 days in culture medium supplemented with 100 ng/ml (h)M-CSF in plates. After harvesting and seeding, BMDM were
polarized for 16 h toward M1 or M2 phenotype with LPS (20 ng/ml) plus IFNγ (50 ng/ml) or IL-4 (20 ng/ml), respectively.

1.5.2 Isolation of Intestinal Crypts and 3D Organoid Culture
The small and large intestine was flushed with cold PBS, cut longitudinally and into 5 mm pieces. To dissociate the crypts, pieces were placed in 25mM EDTA/PBS for 1h at 4°C and suspended in PBS. Isolated crypts were washed, counted and pelleted. A total of 200 crypts were mixed with 40 μl of Matrigel and plated in 24-well plates (Sato et al. 2009). After polymerization of Matrigel, 500 μl of IntestiCult Organoid Growth Medium (Stem Cell) containing Wnt-3a (50 ng) was added. Medium was changed every 3 days. For passage, organoids were removed from Matrigel and mechanically dissociated into single-crypt domains, and then transferred to fresh Matrigel. Passage was performed once per week with a 1:2 split ratio.

1.6 In vivo models of colorectal cancer
1.6.1 AOM DSS model of colitis associated to cancer (CAC)
6-8-week-old wild type and Gpr35−/− mice were injected i.p. with azoxymethane (12.5 mg/kg, AOM). Colitis was induced by two cycles of 2.5% dextran sodium sulfate (DSS) in drinking water for 5 days, followed by a 16 day tap water period (Greten et al., 2004). The final DSS cycle (2%) was administered for 4 days, followed by a 10 day tap water period. The size and number of colonic tumours were evaluated at day 61 microscopically in longitudinally cut and formalin fixed specimen. The typical features of the AOM DSS model are depicted in Table 3.

1.6.2 AOM model of colorectal cancer
AOM (40 mg/kg in total, i.p.) was administered in mice at the single dose of 10 mg/kg at the beginning of the first, second, third and fourth week. The drugs were given i.p. three times a week starting one week before the first administration of AOM. All animals were euthanized by asphyxiation with CO2 three months after the first injection of AOM. Based on our laboratory experience, this time (at the used dose of AOM) was associated with the occurrence of a significant number of aberrant crypt foci (ACF, which are considered pre-neoplastic lesions), polyps and tumours (Izzo et al. 2008) (see table 3 for the AOM model features).

For ACF, polyps and tumours determination, the colons were rapidly removed after sacrifice, washed with saline, opened longitudinally, laid flat on a polystyrene board and fixed with 10% buffered formaldehyde solution before staining with 0.2% methylene blue in saline. Colons were examined using a light microscope at 20X magnification (Leica Microsystems, Milan Italy). The detection and quantization of ACF, polyps and tumours on the colon were performed as previously reported (Izzo et al., 2008). Briefly, in comparison to normal crypts, aberrant crypts have greater size, larger and often elongated openings, thicker lining of epithelial cells, compression of adjacent
crypts, and are more darkly stained with methylene blue. The criterion to distinguish polyps from tumours was established considering the main characteristic features of these two lesions (i.e. crypt distortion around a central focus and increased distance from luminal to basal surface of cells for polyps and high grade of dysplasia with complete loss of crypt morphology for tumours) (Izzo et al., 2008).

URB602 (MAGL inhibitor), AM630 (NAAA inhibitor) and PEA were intraperitoneally administrated at doses of 5 mg/kg, 20 mg/kg and 10 mg/kg, respectively.

In some experiments, MAGL-ko mice were used. In this set of experiments, AOM (40 mg/kg in total, i.p.) was administered at increasing doses (four doses of 1, 2, 3 and 4 mg/kg in a week) during the first, second, third and fourth week. This protocol of administration was selected on the basis of preliminary experiments, in which MAGL-deficient mice were found to be more sensitive the AOM action.

1.6.3 APC\textsuperscript{min} model of sporadic colon cancer

APC\textsuperscript{min} mice were obtained from the Jackson Laboratory and crossed with mice lacking gpr35 globally or conditionally in their intestinal epithelial cells. At the age of 15 weeks mice were sacrificed and after flushing with PBS the intestine was longitudinally cut, formalin fixed and tumours count was assessed microscopically. Samples were embedded as swiss rolls in paraffin for further analysis. APC\textsuperscript{min} model of sporadic colon cancer main characteristics are reported in table 3.

1.6.4 Xenograft model of colon cancer

The effect of URB602 (a MAGL inhibitor) was evaluated on tumour formation induced by subcutaneous injection of HCT116 cells (2.5x10\textsuperscript{6}) into the right flank of athymic female mice for a total volume of 200 µl [50% cell suspension in phosphate buffer solution (PBS) and 50% Matrigel\textsuperscript{TM}] (see table 3 for xenograft tumour features). Ten days after inoculation (once tumours had reached a size of 250-300 mm\textsuperscript{3}), mice were randomly assigned to control and treated groups, and treatments were initiated. Tumour size was measured every day by digital caliper, and tumour volume was calculated according to the modified formula for ellipsoid volume (volume = π/6 × length × width\textsuperscript{2}). Mice were euthanized when the endpoint tumour volume was of 2000 mm\textsuperscript{3}. The MAGL inhibitor URB602, at the dose of 5 mg/kg, was given intraperitoneally (i.p.) every day for all the duration of the experiment. URB602 dose was selected on the basis of previous published work which showed selective inhibitory effects of URB602 on MAGL enzyme without psychoactive effects (Comelli et al. 2007). Xenograft tumour tissue as well as healthy tissue (derived from contralateral paw of the inoculated mice) were collected and analyzed after 8 days of treatment.
The effect of AM9053 (a NAAA inhibitor) and PEA were evaluated on tumour formation induced by subcutaneous injection of HCT116 cells (2.5x10^6) into the back of athymic female mice for a total volume of 200 µL of PBS. The NAAA inhibitor AM9053 at the dose of 20 mg/kg was given peritumorally three times per week for all the duration of the experiment. Tumour size was measured three times per week by digital caliper, and tumour volume was calculated as reported above. AM9053 dose was selected on the basis of previous published work in which AM9053 selectively inhibits NAAA during the experimental colitis (Alhouayek et al. 2015).

1.6.4.1 Xenograft tumour secretome preparation
Nude mice were sacrificed 21 days post-injection, untreated tumours excised and cultured in DMEM (without foetal bovine serum) for 24 h. The conditioned medium was collected and added to Caco-2 cells for 24h at 37°C. Then, the Caco-2 cells were washed in PBS and homogenized in 1.0 mL of TRIzol for further analyses.

1.7 Cell viability (neutral red assay)
Cell viability was evaluated by measuring the neutral red uptake (NR assay). Cells were seeded in presence of 10% FBS in 96-well plates at a density of 1x10^4 cells per well (Caco-2, HCT116 and HCEC cells and allowed to adhere for 24 h). After this period, the human colonic cells were incubated with medium containing 10% FBS in presence or absence of increased concentrations of AM9053 (0.01–10 μM) and PEA (0.1-30 μM) for 24 h. Subsequently, cells were incubated with NR dye solution (50 μg/ml in 10% FBS) for 3 h at 37°C and then lysed with 1% acetic acid. The absorbance was read at 532 nm (iMarkTM microplate reader, Bio-Rad). All results are expressed as percentage of cell viability (n = 3 experiments including 8–10 replicates for each treatment).

1.8 Proliferation assays
1.8.1 BrdU incorporation in colonic epithelial cells
HCT 116, Caco-2 and HCEC were seeded in 96-well plates (1.0x10^4 cells per well), allowed to adhere (within 24h) and starved by serum deprivation for 24h. Cells were then treated with NAAA-inhibitor AM9053 (0.1-3 μM) and PEA (1- 30 μM). After 24 hours of treatment, cells were incubated with BrdU (10 μM) in the cell medium for 2 hours. Thereafter, the proliferation of the cells was determined by using the BrdU proliferation ELISA kit (Roche, Milan, Italy) according to the manufacturer’s instructions. All results are expressed as percentage of cell proliferation (n = 3 experiments including 8–10 replicates for each treatment).

1.8.2 EdU incorporation in colonic organoids
For EdU incorporation, 40 μl of the matrigel containing colonic organoids was seeded in 8-well chamber-slide. 10 μM EdU was added to the wells 6 h prior to fixation. The proliferation marked by
EdU incorporation was detected with Click-iT Edu Imaging Kits (Invitrogen) in according to the supplier’s instructions.

1.8.3 Proliferation assay in HUVEC
For proliferation assay, HUVEC were seeded in 96-well plates at a density of 1x10⁵ cells per well and after adherence (3-4 h) they were serum starved for 12 hours. Cells under basal condition [0.1% fetal calf serum (FCS)] or stimulated for 24 h with either 1% FCS or FGF-2 (20 ng/ml) were treated with URB602 (0.1 and 1 μM). Cells were then fixed, stained and randomly counted at 20 x magnification (Donnini et al. 2006). Data are expressed as counted cells/well.

1.9 Migration assay
1.9.1 Scratch assay in human colon adenocarcinoma cell lines
The migration “wound healing” scratch assay was performed as previously described by Liang and colleagues (Liang et al., 2007). Sub-confluent adenocarcinoma cell line, i.e. HCT116 and Caco-2, were trypsinized and plated into culture-insert two wells (ibidi GmbH, Munich, Germany) inserted on a 24-well plate (condition: 5x10⁴ cells suspended in 70 µl per well of the insert) and leaved to adhere overnight. After this time, the insert was removed, cells were washed with phosphate buffer saline (PBS 1X) and treated with mitomycin C 30µg/ml (Sigma-Aldrich, Milan, Italy) in serum-free media, in order to completely inhibit cell proliferation. After 2 hours, HCT116 and Caco-2 cells were treated with the NAAA-inhibitor, AM9053 (3 µM) or PEA (30 µM) for 24 hours. Wound area recovery was observed under a phase-contrast microscope (iRiS Digital Imaging System, Twin Helix, Rho, Italy) and photographed at the time zero point (right after the mitomycin C removal) and after the 24-hour-treatment. Successively, by using the ImageJ software, the size of the opened area was measured from the digital images. The results are expressed as % of scratch closure (time zero/time 24h*100). Two imagines were acquired for each well and at least 3 replicates were analyzed for each treatment. Three independent experiments were independently carried out.

1.9.2 Scratch assay in HUVEC
HUVEC were seeded into 24-well plates (1 x 10⁵ cells/well) and incubated for 24 h to confluence. Then, the monolayer was scraped using a sterile micropipette tip to create a wound of ± 1 mm width. Cells were washed twice with PBS, and fresh medium containing URB602 (0.1-1 μM was added with ARA-C (2.5 µg/ml) to inhibit cell proliferation. Images of the wound in each well were acquired from 0 to 8 h under a phase contrast microscope at 20 x magnification. After 8 h cells were stained with Hoechst 33342 and image acquisition and analysis were performed by a Nikon digital camera DS-5MC and NIS Element software, using a Nikon Eclipse TE 300 inverted microscope (Nikon, Tokyo, Japan). Results are expressed as percentage of area of wound (Monti et al. 2010).
1.10 Angiogenesis analysis
1.10.1 Tube formation assay
SVEC4-10 cells were stained with calcein AM for 30 min at 37°C. After counting, SVEC4-10 cells were seeded (7x10^4 cells/well) on growth factor-reduced matrix (Geltrex) -coated 24-well plates and were cultured for 6h with conditioned medium of M2-polarized macrophages. Pictures of tube formation were acquired at confocal microscope and Angiogenesis Analyzer for ImageJ was used for quantification of tube networks.

1.10.2 CXCL1/KC detection
CXCL1/GRO alpha, also known as KC in mouse and CINC-1 in rat, is a member of the CXC family of chemokines. KC was detected in macrophages supernatants by using Quantikine Mouse KC Immunoassay ELISA (R&D system).

1.10.3 Aortic ring assay
Murine thoracic aortas were collected from mice lacking GPR35 or their wildtype littermates. After flushing and removing the connective tissue, aortas were cut into 0.5-1 mm long rings and serum-starved overnight at 37 °C and 5% CO2 in Opti-MEM. Then the aortic rings were individually embedded in rat type I collagen (Millipore, MA, USA), feed with Opti-MEM supplemented with 2.5% FBS and 30ng/mL VEGF. The aortic rings from GPR35+/+ and GPR35−/− mice were treated with vehicle or CXCL17 (0.2mg/mL). The growth medium was changed on days 3, 6 and 8. On day 8 the number of microvessels sprouting was counted using the microscope (Baker et al., 2011).

1.10.4 Quantification of angiogenesis modulators in HUVEC by ELISA
FGF-2 and endostatin levels were measured in conditioned media by ELISA kits (R&D System, Minneapolis, MN, USA). HUVEC were exposed to URB602 (1 µM, 18 h) in 0.1% or 1% FCS. Cell culture supernatants were collected, stored at −80°C and analysed following manufacturer instructions. Data are reported as pg/mL (FGF-2) or ng/mL (endostatin) (means ± SEM). Experiments were run in triplicate.

1.11 Evaluation of metabolic profile
1.11.1 Glucose uptake
BMDM were seeded (5x10^4 cells/well) in white 96-well plates, polarized for 16h as described above or used as M0 macrophages. We used a luminescence-based glucose uptake assay (Glucose Uptake Glo, Promega, USA). Cells were washed once with PBS and then incubated with PBS containing 1 mM 2-deoxy-D-glucose (2-DG) for 60 min. The reaction was stopped at different time points and the detection reagent for 2-deoxy-D-glucose-6-phosphate (2DG6P) was added. Luminescence was measured thereafter. We used a standard curve of 2DG6P to extrapolate the concentration of 2DG6P in the sample and calculate the rate of glucose uptake over time per cell.
1.11.2 Seahorse extracellular flux analysis
BMDM were seeded (7.5x10^4 cells/well) in 96-well XFe Seahorse plates, differentiated for 16 h as described above or left undifferentiated. 1 hour prior to the start of extracellular flux measurements, the medium was changed to Agilent Seahorse XF Base Medium supplemented with 1 mM L-glutamine and 10 mM D-glucose. Medium pH was set to 7.4 after warming to 37 °C. Oxygen consumption rate (OCR) was measured using the XFe96 Seahorse extracellular flux analyser (Agilent, USA) over the course of 2 hours. Oligomycin (1 µM), FCCP (1.5 µM) and Rotenone/Antimycin A (both 1 µM) were injected sequentially to allow assessment of respiratory chain functionality. Glycolysis was assessed through measurement of extracellular acidification rate (ECAR) after sequential injections with D-glucose (10 mM), Oligomycin (1 µM) and 2-deoxyglucose (100 mM).

1.11.3 Lactate measurements
BMDM were seeded (5x10^4 cells/well) in dialyzed medium in 96-well plate, differentiated for 16h as described above. Lactate concentration was measured in 5 µL of media collected at experimental time points (12, 24 and 48h) using a luminescence based assay (Lactate Glo, Promega) in according to the supplier’s recommendations. Plates were mixed briefly and incubated for 1h at 37°C before reading luminescence. Lactate concentration was determined by comparison to a lactate standard curve.

1.12 BrdU incorporation and quantification
BrdU at dose of 2.5 mg/kg was injected i.p 24 h before euthanizing mice. Paraffin embedded samples were then de-paraffinised in xylene and de-hydrated with ethanol. Cells positive for BrdU were detected using a BrdU staining kit (BD). Proliferation was revealed as BrdU+ cells along the crypt–villus.

1.13 Identification and quantification of endocannabinoids, PEA and related molecules
Endocannabinoids [anandamide (AEA) and 2-arachidonoylglycerol (2-AG)], palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) levels were measured in tumour and healthy tissues of xenografted mouse as well as in HCT116 cells (used for inoculation) by isotope dilution liquid chromatography-atmospheric pressure-chemical ionization mass spectrometry as previously described (Di Marzo et al., 2008). Briefly, collected samples were immediately immersed into liquid nitrogen and stored at -80°C until extraction of endocannabinoids (AEA and 2-AG), PEA and OEA. Samples were extracted with chloroform/methanol (2:1, by volume) containing each 5 pmol of d8-anandamide, d4-PEA, d4-OEA and d5-2-AG, synthesized as described previously (for the former two compounds) or provided by Cayman Chemicals (for d5-2-AG, Ann Arbor, MI). The lipid extracts were purified by silica column chromatography and the
fractions containing anandamide, PEA, and 2-AG were analyzed by isotope dilution liquid chromatography–atmospheric pressure–chemical ionization mass spectrometry. Results were expressed as picomoles per milligram of lipid extract.

1.14 Immunohistochemistry

The animals were deeply anesthetized with pentobarbital (100 mg/kg) and then perfused transcardially with 0.9% saline solution for 5 min, followed by 100ml of fixative containing 4% paraformaldehyde (wt/vol)/0.1M phosphate buffer (pH 7.4). After fixation tumour slices were cut with a Leica CM3050S cryostat in serial frozen sections (10 μm-thick) and processed for immunofluorescence. Immunohistochemical study of MAGL expression in tumour slices collected from xenografted mice was performed by incubation of sections with goat anti-MAGL (1:200, Abcam, Milan, Italy) revealed by specific Alexa-594 secondary donkey anti-IgGs antibody (Invitrogen Life Technology, Paisley, UK) and counterstained with 4',6-diamidino-2-phenylindole. Immunoreactivity for MAGL was analyzed by DMI6000 microscope equipped with appropriate filters and deconvolution software MetaMorph LAS AF 2.2.0 software (Leica, Germany).

1.15 Immunostaining for CD31

Collected xenograft tumours were immediately embedded in Tissue-Tek O.C.T. (Sakura, San Marcos, CA), cooled in isopentane and frozen in liquid nitrogen for immunostaining and histological analysis. Seven μm-thick cryostat sections from a xenograft tumour were stained with hematoxylin and eosin and adjacent sections were used for immunohistochemical staining with anti CD31 (Chemicon, Millipore, Milan, Italy) antibody. For immunohistochemistry, cryostat sections were firstly fixed in acetone at -20°C and incubated for 10 min in 3% H2O2, washed (3 x 5 min) in Tris-buffered saline (TBS) and then incubated in a Blocking reagent (KIT Immunoperoxidase Secondary Detection System, Chemicon, Millipore, Milan, Italy). Mouse monoclonal anti-CD31 antibody diluted 1:100 in TBS, 0.05% bovine serum albumin (BSA) was applied. Negative controls were produced by eliminating the primary antibodies from the diluents. Sections were then washed (3 x 5 min in TBS) and incubated for 10 min in the appropriate species–specific biotinylated secondary antibodies (goat anti mouse IgG, KIT Immunoperoxidase Secondary Detection System, Chemicon). Following washings (3 x 5 min in TBS), the sections were incubated for 10 min in streptavidin-conjugated HRP and then exposed to 3,3-diaminobenzidine tetrahydrochloride (DAB, detection kit, Millipore, Milan, Italy) for 8 min to produce a brown reaction product. Finally, sections were counterstained in hematoxylin and mounted in Aquatex (Merck, Milan, Italy) (Finetti et al. 2012).
For CD31 immunofluorescence labelling, sections were fixed in acetone, and aspecific binding sites were blocked by using 3% BSA. Slides were incubated with a rat anti-mouse CD31 antibody (Millipore, Billerica, USA). Immunoreactions were revealed by using FITC anti-rat secondary antibodies. Images were analyzed using Nikon Eclypse T200, and quantification of human CD31 and vessel lumina were performed counting 10 random field/section for slides.

1.16 Gene expression analysis by quantitative Real-Time (q)-PCR
Healthy tissues and tumours collected from both xenografted mice as well as from patients were immediately immersed into RNA Later and stored at -80°C until RT-PCR analysis. RNA from macrophages was isolated using the RNeasy Mini kit (QIAGEN). RNA was then reverse transcribed with M-MLV RT (Invitrogen), and SYBR-Green (Eurogentec) qPCR was performed using MX-3000 (Agilent Technologies).

Tumours and colorectal cancer cells were homogenized in 1.0 mL of TRIzol (Invitrogen) following the manufacturer’s instructions. Total RNA from murine and human tissues was purified, quantified, characterized and retrotranscribed as previously described (Iannotti et al., 2010). Final preparation of RNA was considered DNA- and protein-free if the integrity number (Bionalyzer 2100, Agilent) was greater than 8 relative to a 0–10 scale. Quantitative real-time PCR was carried out in a iCycleriQ5 system (Biorad, MI, Italy) by use of SYBR Green detection. Selective primers were designed using Allele-Id software version 7.0 (Biosoft International, Palo Alto, CA, USA) and synthesized by MWG-Biotech (HPLC purification grade) (see Table 4). Each sample was amplified simultaneously in a quadruplicate in one-assay run (maximum DCt of replicate samples <0.5), and a standard curve from consecutive fivefold dilutions (100–0.16 ng) of a cDNA pool representative of all samples was included for PCR efficiency determination. Data normalization was performed by using as a control the ct from S16 and/or HPRT, both constitutively expressed proteins; differences in mRNA content between groups were calculated as normalized values by use of the 2^(-ΔΔct) formula (Iannotti et al., 2010).

1.17 Western blot analysis
The expression of vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2) and endostatin was assessed in xenograft tumour tissue of animals treated or not with URB602, the MAGL inhibitor. Samples were fragmented in liquid nitrogen, lysed in Cell Lytic buffer (Invitrogen) and centrifuged at 14000 rpm for 15 min. Supernatant was collected and protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad); lysate aliquots containing 50 µg of proteins were separated by SDS-PAGE (4-12% PA, NuPAGE, Invitrogen) and transferred to a nitrocellulose membrane using iBlot2 (Life Technologies, Monza, Italia). After blocking in 5% w/v nonfat dry milk (1X PBS) buffer, membranes were incubated overnight with anti-FGF-2
(1:1000, Upstate), polyclonal anti-VEGF (1:1000, Millipore) or anti-endostatin (1:1000, Millipore); after washing, secondary antibody anti-mouse or anti-rabbit IgG (1:2500, Promega), linked to horseradish peroxidase, was added. The signal was visualized by enhanced chemiluminescence using Chemidoc XRS (Biorad) and analyzed using Quantity One Software version 4.6.3. The membranes were probed with anti-β-actin antibody (1:1000, Sigma) to normalize the results, which were expressed as ratio of densitometric analysis of each protein/β-actin bands.

1.18 Statistical analysis
Data are expressed as the mean ± SEM or SD of n experiments. To determine statistical significance, Student's t-test was used for comparing a single treatment mean with a control mean, and a one-way ANOVA followed by a Tukey multiple comparisons test was used for analysis of multiple treatment means. P-value < 0.05 was considered to be significant. G Power was used for sample size calculation.
**TABLE 3 Mouse models of colorectal cancer**

<table>
<thead>
<tr>
<th>Model</th>
<th>Primary location</th>
<th>Invasiveness</th>
<th>Driver</th>
<th>Sporadic CRC vs CAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC&lt;sup&gt;+/–&lt;/sup&gt;</td>
<td>Small intestine</td>
<td>Adenomas</td>
<td>Mice are heterozygous for an <em>Apc</em> loss-of-function mutation Increased WNT–β-catenin signalling occurs following allelic loss of wild-type <em>Apc</em></td>
<td>Sporadic CRC</td>
</tr>
<tr>
<td>Xenografts</td>
<td>Usually subcutaneous or orthotopic</td>
<td>Variable</td>
<td>Variable</td>
<td>NA</td>
</tr>
<tr>
<td>DSS-AOM</td>
<td>Colon</td>
<td>Aberrant crypt foci and adenomas</td>
<td>Intestinal barrier dysfunction and inflammation driven by the chemical irritant DSS, combined with AOM-induced mutagenesis</td>
<td>CAC</td>
</tr>
<tr>
<td>AOM</td>
<td>Colon</td>
<td>Aberrant crypt foci, polyps and adenomas</td>
<td>AOM-induced mutagenesis</td>
<td>Sporadic CRC</td>
</tr>
</tbody>
</table>

(adapted from West et al., 2015)

AOM, azoxymethane; APC, adenomatous polyposis coli protein; CAC, colitis-associated cancer; CRC, colorectal cancer; DSS, dextran sodium sulfate; NA, not applicable.
**TABLE 4. Sequences of primers. List of genes screened by Quantitative Real-Time Polymerase Chain Reaction.**

<table>
<thead>
<tr>
<th>Targeted gene</th>
<th>FORWARDED Sequence (5'-&gt;3')</th>
<th>REVRED Sequence (5'-&gt;3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sequences for Homo sapiens primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAAH</td>
<td>GGCGGAGTGCCACAGCGTAG</td>
<td>GCCACTCTTGCTGAGGCGGT</td>
</tr>
<tr>
<td>PPAR-α</td>
<td>TTCGGATCCATCGGCGG</td>
<td>CCACAGGATAGTCACCGAGG</td>
</tr>
<tr>
<td>TRPV1</td>
<td>CCTGCGTCTAGCTGGTACA</td>
<td>CTTGGGACAGCACCTGGCAC</td>
</tr>
<tr>
<td>CB1</td>
<td>TCTGGTCTACATGTCATGC</td>
<td>CTTGGCTAACTAATGTC</td>
</tr>
<tr>
<td>CB2</td>
<td>TAGTGCTAGAGGAGC</td>
<td>CGCTATCCACCTTCATCAA</td>
</tr>
<tr>
<td>NAAA</td>
<td>TTAAAGAATGCGCCAGATT</td>
<td>CTTTATACCTGGTCA</td>
</tr>
<tr>
<td>GPR55</td>
<td>GAAAACCTACATTGCGATTC</td>
<td>GAGGTGCACCATATAATGGG</td>
</tr>
<tr>
<td>MAGL</td>
<td>GCCAGGAGCAGAGCGAAG</td>
<td>CCAGAAGAAGACAGAAGCC</td>
</tr>
<tr>
<td>p27kip</td>
<td>ACGCGAGATCTTCTACTCA</td>
<td>CTTCTGGCCCTTACTG</td>
</tr>
<tr>
<td>VEGF</td>
<td>CTTTGCTCTCTTTATTTGTA</td>
<td>TAGTTATGAGATGTATTAGAA</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>GACCTTCGTTGCCCTCTG</td>
<td>AGGGAGTGAAGTGATTTTCG</td>
</tr>
<tr>
<td>β-actin</td>
<td>GGCTTACTTCAGGGTGAGGA</td>
<td>GATGCCTTCCCCGAGCCGTCTTT</td>
</tr>
<tr>
<td>RNA POL</td>
<td>ACGACTTTTGATGATGG</td>
<td>GGAGATTCGACATCTC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGAGCGAGACTCCCTCCAAATG</td>
<td>GCCTGTCATACACTTCATTC</td>
</tr>
<tr>
<td>GPR35</td>
<td>GCTCAGCCACAGGCTTTCCTC</td>
<td>TAGTTGCACATCATGGTCCTTC</td>
</tr>
<tr>
<td><strong>Sequences for Mus musculus primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAAH</td>
<td>ACTTTGACGTTGCTTAAAACC</td>
<td>GCCTTACCCCTTTTCAATGC</td>
</tr>
<tr>
<td>PPAR-α</td>
<td>AGAGCAGCTTCTGCTCT</td>
<td>ACTGTTAGCTGCAAAAACCAA</td>
</tr>
<tr>
<td>TRPV1</td>
<td>CCACCTGTTGAGACGCC</td>
<td>TCTGGGCTTCTGAAAATTGG</td>
</tr>
<tr>
<td>CB1</td>
<td>GGGCAGCTTCGACTGCTTC</td>
<td>GTGGAAGTGAAGGTATTTTC</td>
</tr>
<tr>
<td>CB2</td>
<td>AGAAGATGCTGCTAGGTCATTC</td>
<td>ATGTTAGCTAGGTCATTC</td>
</tr>
<tr>
<td>NAAA</td>
<td>CCAAGACTCTCCCTTACATG</td>
<td>CTACAGCTGAAACTCTC</td>
</tr>
<tr>
<td>GPR55</td>
<td>CAGCCGATCCCCATATCCCAC</td>
<td>GCACCAGAATAAACCCAA</td>
</tr>
<tr>
<td>GPR35</td>
<td>AAATCCCCACCTTTTCAGAAC</td>
<td>CATGTTGACAGGTACATAG</td>
</tr>
<tr>
<td>GLUT1</td>
<td>TGGCGGAGACAGCGATATT</td>
<td>AACTCCCTAATAACCCTTTTCAG</td>
</tr>
<tr>
<td>SGLT1</td>
<td>ACGTCGCACTTGTACCGTTG</td>
<td>CTTTATCCCTGCTGACG</td>
</tr>
<tr>
<td>SGLT2</td>
<td>GTCTTTGCTTGTTGCTTACATG</td>
<td>TCACACTTAAATCCAGG</td>
</tr>
<tr>
<td>β-actin</td>
<td>GCCCTAGGCCCTCTTTCAG</td>
<td>TGCCACAGAGATCCCTAC</td>
</tr>
</tbody>
</table>

Materials and Methods
RESULTS

Chapter I. Pharmacological inhibition of MAGL reduces experimental colon tumorigenesis


1.1 MAGL expression in human colorectal carcinoma (HCT 116) cells and xenograft tumour tissues

In order to elucidate the role of MAGL in colorectal tumorigenesis, we generated xenograft tumours by inoculating human colorectal cancer (HCT116) cells in athymic mice. Human MAGL mRNA was weakly expressed in HCT116 cells, but it displayed about 5-fold increase in the xenograft tumour tissue (Figure 13A). MAGL was undetectable in the mouse controlateral paw, used as negative control (data not shown). Immunostaining revealed high expression of MAGL protein in tumour tissues (Figure 13B), although the precise localization of the enzyme within the cell was not detectable.

1.2 The MAGL inhibitor URB602 reduces tumour growth in the xenograft model of colon cancer

We evaluated the potential antitumoral effect of the MAGL inhibitor URB602 by using a xenografted tumour model generated as described above. On day 10 after s.c. cells inoculation, all mice developed tumours, with a mean volume (± SEM) of 288±37 mm³. Thereafter, mice were treated daily either with vehicle or URB602 (5 mg/kg, i.p.) until the sacrifice of the animals (i.e. after eight days). As shown in Figure 14, URB602 significantly reduced tumour growth compared to vehicle. Eight days after drug (or vehicle) administration, the average tumour volume in the control group was 1980±269 mm³, whereas the average tumour volume in the URB602-treated group was 956±180, exhibiting a 52% of tumour growth inhibition (Figure 14).

1.3 Effect of URB602 (a MAGL inhibitor) on 2-AG levels in healthy and tumour tissues from xenografted mice

The levels of 2-AG found in tumour tissues of xenografted mice were higher than those detected in CRC (HCT116) cell line (Figure 15A, insert) and in healthy tissues derived from the contratralateral paw of xenografted mice (Figure 15A). URB602, at the daily i.p. dose of 5 mg/kg, significantly increased, by approximately four-folds, the levels of 2-AG in healthy tissues, but it was not able to further increase the already elevated 2-AG levels in tumour tissues (Figure 15A).

In order to verify URB602 selectivity vs its target (i.e. MAGL), we measured the levels of anandamide and related acylethanolamides following URB602 treatment. The MAGL inhibitor did not significantly change anandamide levels in both healthy and tumour tissues (Figure 15B).
Similarly, levels of PEA and OEA, both in healthy (PEA pmol/mg: control: 105.6±46.64, URB602: 220.2±120.1; OEA pmol/mg: control: 11.02±3.86, URB602: 23.12±6.447; mean ± S.E.M., n=5 mice for each experimental group) and in tumour (PEA pmol/mg: control: 121.1±41.79, URB602: 124.5±44.19; OEA pmol/mg: control: 12.0±1.597, URB602: 9.50±2.132; mean ± S.E.M., n=5 mice for each experimental group) tissues, were unchanged. Collectively these results suggest the selectivity of URB602 for MAGL vs acylethanolamide-hydrolyzing amidases.
Results – Chapter I
Pharmacological inhibition of MAGL reduces experimental colon tumorigenesis

Figure 13. Expression of MAGL in the xenograft model of colon cancer.
(A) Human MAGL mRNA was evaluated by quantitative (real-time) RT-PCR analysis in human colorectal cancer (HCT 116) cells and in xenograft tumour tissues (mean ± SEM of 5 independent experiments). Data were not statistically analyzed because cells are phenotypically different.
(B) Immunohistochemical analysis of MAGL in tumour tissues (xenograft model of colon cancer).
Figure 14. Inhibitory effect of URB602 (a MAGL inhibitor) on tumour formation in the xenograft model of colon cancer.

Tumours were induced by subcutaneous injection of colorectal cancer (HCT 116) cells into the right flank of athymic female mice. Tumour size was measured every day by a digital caliper, and tumour volume was calculated. URB602 (5 mg/kg, i.p.) treatment started 10 days after cell inoculation and continued daily until the sacrifice of the animals. Results represent the mean ± SEM of 9–11 mice. *p < 0.05 and ***P < 0.001 vs control (vehicle-treated nude mice).
Pharmacological inhibition of MAGL reduces experimental colon tumorigenesis

Figure 15. Effect of URB602 (a MAGL inhibitor) on endocannabinoids levels in the xenograft model of colon cancer.

2-arachydonylglycerol (2-AG, A) and anandamide (AEA, B) levels were evaluated in healthy tissues [derived from contralateral flank of the xenografted mice (i.e. the flank not injected with xenografted cells)] and in the tumour tissue. The inserts show a comparison between the levels of 2-AG (insert to A) and anandamide (insert to B) in colorectal cancer (HCT116) cells and in xenograft tumour tissue. Data of the insert were not statistically analyzed because cells are phenotypically different. URB602 (5 mg/kg, i.p.) treatment started 10 days after cell inoculation and it was administered every day until the sacrifice of the animals. Assays were performed 8 days after the first injection of URB602 (i.e. the day of animal sacrifice). Results are mean ± SEM of 5 independent experiments for each experimental group. *P < 0.05 vs vehicle-treated nude mice.
1.4 The MAGL inhibitor URB602 drives in vivo angiogenesis output in xenograft tumour tissues

Figure 16 shows a panel of angiogenesis modulators following URB602 treatment. URB602 (5 mg/kg daily) decreased the expression of pro-angiogenic factors (i.e. VEGF and FGF-2), while it had no effect on the expression of endostatin, an anti-angiogenic modulator in tumour tissues. We also evaluated, by RT-PCR, mRNA expression of VEGF, the most important pro-angiogenic factor (Shibuya et al., 2011; Ferrara, 2004). Results, depicted in the insert to Figure 16B, showed that URB602 treatment decreased VEGF mRNA transcription. Tumour samples were also analyzed for microvessel density (by counting CD-31 positive neovessels), structure and composition. All these parameters were significantly reduced by URB602 (Figure 17). Collectively, such results suggest antiangiogenetic effects in vivo.

1.5 Direct antiangiogenic effect of the MAGL inhibitor URB602 in HUVEC

To verify if URB602 exerts a direct antiangiogenic effect, the MAGL inhibitor was tested in HUVEC stimulated with FGF-2 or 1% FCS. Both FGF-2 or 1% FCS (24 h stimulation) increased cell proliferation (Figure 18). The stimulated cell proliferation was inhibited by non-cytotoxic concentrations of URB602 (0.1 and 1 µM) (Figure 18). In un-stimulated cells (0.1% FCS), URB602, at the 0.1 µM concentration, stimulated proliferation (Figure 18). Concentrations higher than 1 µM were not used because they resulted in a cytotoxic effect. The antiangiogenic effect of URB602 was corroborated by the observation that the inhibitor, at the 1 µM concentration, significantly reduced migration of adherent cells induced by both FGF-2 and 1% FCS (8 h incubation) (Figure 19). Specifically, the angiogenic factors reduced wound area and URB602 impaired the closure of wounds [see the quantification of scratch reported as percentage of healing in Figure 19 (bottom) and the representative pictures in Figure 19 (top)]. In un-stimulated cells (0.1% FCS), URB602 increased migration (Figure 19). Finally, we analyzed the balance of pro- and anti-angiogenic factors released by HUVEC in the culture media. The increase of FCS concentration (from 0.1% to 1%) induced a significant increase in FGF-2 release and a significant decrease in endostatin levels, as a sign of endothelial activation (Figure 20, see white columns). Both these changes were restored by URB602 treatment (Figure 20). Collectively, such data suggest a direct effect of URB602 on endothelial cells, through the modulation of endothelial cells functional response and production of autocrine/paracrine modulators of angiogenesis.

1.6. The MAGL inhibitor URB602 regulates Cyclin D1 in xenograft tumour tissues

In order to investigate the antiproliferative effects of URB602 in vivo, the expression of two cell cycle regulator genes (i.e. cyclin D1 and p27KIP) was evaluated in tumour xenograft tissues.
URB602 decreased the mRNA expression of cyclin D1 (Figure 21A), a factor that, by promoting cell DNA synthesis and cell growth, stimulates cancer cell proliferation (Motokura and Arnold, 1993). No significant effects of URB602 on the cycle-negative regulator p27KIP was observed (Figure 21B).

1.7. **URB602 exerts chemopreventive effects in the azoxymethane (AOM) model of colon carcinogenesis**

The carcinogenic agent AOM up-regulated colonic mRNA MAGL expression (mRNA fold expression of MAGL±SEM: control: 1.0±0.06, AOM: 1.7±0.04*; n=4 *p<0.05 vs control) and induced the expected appearance of ACF (Figure 22A), polyps (Figure 22B) and tumours (Figure 22C) after a three months treatment. URB602 significantly reduced AOM-induced ACF (Figure 22A) and polyps (Figure 22B), while it showed a trend to decrease the number of polyps (Figure 22B). URB602 (5 mg/kg), given alone (i.e. in absence of AOM), did not induce ACF, polyps and tumours, thus demonstrating that this compound is devoid of tumour-initiating activity in this experimental model (data not shown).

URB602 did not modify significantly AOM-induced ACF (Figure 22A), polyps (Figure 22B) and tumours (Figure 22C) in MAGL-deficient mouse, confirming the MAGL involvement in its antitumour activity.
Figure 16. Effect of URB602 (a MAGL inhibitor) on angiogenic factors in the xenograft model of colon cancer.

The effect of URB602 was evaluated on VEGF (A), FGF-2 (B), and endostatin (C) expression in the tumour mass. URB602 (5 mg/kg, i.p.) treatment started 10 days after cell inoculation and it was administered every day until the sacrifice of the animals. Assays were performed 8 days after the first injection of URB602. (A) Representative blots, (B–D) results expressed as ratio of densitometric analysis of each protein/β-actin bands (means ± SEM of 3 tumours from three different mice. Each experiment was in triplicate). ***P < 0.001 vs vehicle-treated nude mice. The insert shows the mRNA expression of VEGF in the tumour tissue (HCT116 xenograft model) from animals treated or not by URB602. *P < 0.05 vs vehicle-treated nude mice.
Pharmacological inhibition of MAGL reduces experimental colon tumorigenesis.

Figure 17. Effect of URB602 (a MAGL inhibitor) on CD31 positive cells in the xenograft model of colon cancer.
Representative images of CD31 immunostaining (upper) or immunofluorescence (bottom) in tumour sections from untreated (vehicle) and URB602-treated mice (URB602). Scale bars indicate 100 (upper) or 50 (bottom) μm. Images obtained by fluorescence microscope (Eclipse TE300, Nikon) at 20× (upper) or 40× (bottom) magnification and taken by a digital Q10 camera. Bar graph represents the num-ber of CD31 positive vessels, calculated counting 8 random fields/section for slides, each slide having five sections. URB602 (5 mg/kg, i.p.) treatment started 10 days after cell inoculation and it was administered every day until the sacrifice of the animals. Assays were performed 8 days after the first injection of URB602. Results are mean ± SEM tumours from three different mice. Each experiment was in triplicate. ***P < 0.001 vs vehicle-treated nude mice.
Figure 18. Effect of URB602 (a MAGL inhibitor) on endothelial cell proliferation *in vitro*. 
URB602 was tested on cell proliferation in human umbilical vein endothelial cells (HUVEC) under basal condition (0.1% FCS) or in cells stimulated for 24 h with either 1% FCS or FGF-2 (20 ng/ml).

(A) URB602 (0.1–1 \( \mu \text{M} \)) was added to the cell media 30 min before stimulation. Data are expressed as counted cells/well ± SEM from 4 independent experiments. **\( p < 0.01 \) and ***\( p < 0.001 \) vs 0.1% FCS URB602-untreated cells, §\( p < 0.05 \) and §§§\( p < 0.001 \) vs 1% FCS URB602-untreated cells; ##\( p < 0.01 \) vs FGF-2 URB602-untreated cells.
Pharmacological inhibition of MAGL reduces experimental colon tumorigenesis

Figure 19. Effect of URB602 (a MAGL inhibitor) on endothelial cell migration in vitro.
URB602 was tested on cell migration in human umbilical vein endothelial cells (HUVEC) under basal condition (0.1% FCS) or in cells stimulated for 24 h with either 1% FCS or FGF-2 (20 ng/ml). Bottom: effect of URB602 (1 μM) in HUVEC monolayers. URB602 was added to the cell media 30 min before stimulation. The graph shows the percentage of healing of the wound area. *p<0.05 and ***p<0.001 vs. 0.1% FCS URB602-untreated cells, #p<0.05 vs. 1% FCS URB602-untreated cells and §§§p<0.001 vs. FGF-2 URB602-untreated cells.
Top: Representative pictures (of 4 experiments) of Hoechst 33342 labelled cells.
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Pharmacological inhibition of MAGL reduces experimental colon tumorigenesis

Figure 20. Effect of URB602 (a MAGL inhibitor) on angiogenic factors in vitro.
URB602 (1 μM) was tested on FGF-2 (A) and endostatin levels (B) in human umbilical vein endothelial cells (HUVEC) under basal condition (0.1% FBS) or in cells stimulated for 18 h with either 1% FBS. Data are reported as pg/ml (FGF-2) or ng/ml (endostatin) (means ± SEM) from 3 independent experiments (in triplicate). ***p<0.001 vs. 0.1% FBS URB602-untreated cells, ##p<0.01 and ###p<0.001 vs 1% FBS URB602-untreated cells.
Pharmacological inhibition of MAGL reduces experimental colon tumorigenesis

Figure 21. Effect of URB602 (a MAGL inhibitor) on expression of cycle regulator genes in the xenograft model of colon cancer.

Cyclin D1 (A) and P27kip (B) mRNA expression were evaluated by quantitative (real-time) RT-PCR analysis in the tumour mass of vehicle- and URB602-treated mice. URB602 (5 mg/kg, i.p.) treatment started 10 days after cell inoculation and it was administered every day until the sacrifice of the animals. Assays were performed 8 days after the first injection of URB602. *P < 0.005 vs vehicle-treated nude mice.
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Figure 22. Azoxymethane (AOM) model of colon carcinogenesis: effect of URB602 (a MAGL inhibitor) in wild type and in Mgl\(^{-/-}\) deficient mice.

The inhibitory effect of URB602 was evaluated on aberrant crypt foci (ACF) (A), polyps (B) and tumours (C) induced in the wild type (left) and MAGL-deficient (right) mice by AOM. URB602 (5 mg/kg, i.p.) was given three times a week for the whole duration of the experiments starting 1 week before the first administration of AOM. Measurements were performed 3 months after the first injection of AOM. The criterion to distinguish polyps from tumours was established considering the main characteristic features of these two lesions. Results represent the mean ± SEM of 9–11 mice.

*p < 0.05 and **p < 0.01 vs AOM alone
Chapter II. NAAA is crucially involved in proliferation, migration and *in vivo* colon tumorigenesis

1.1 NAAA is down-regulated in human CRC tissues

Using a web accessible database named GENT (available at [http://medicalgenome.kribb.re.kr/GENT](http://medicalgenome.kribb.re.kr/GENT)), which provides the gene expression patterns across different kind of human cancer and healthy tissues, we found that hNAAA enzyme is significantly downregulated in colonic biopsies of patients with colorectal cancer compared with corresponding normal tissues (Figure 23A). Therefore, we examined the gene expression of hNAAA in colonic tumours of patients at different stages of CRC by RT-PCR. As shown in Figure 23B, a significant reduction of NAAA was observed in tumour specimens (compared to their corresponding non-cancerous controls) at different stages (pT2, pT3 and pT4 of CRC). We also measured the expression of PPAR-α mRNA, i.e. the main target of PEA, in CRC biopsies.

We found that PPAR-α mRNA was differently expressed in CRC tissues with a trend toward a reduction in patients with stage pT3 and pT4 tumours compared to normal tissues (Figure 23C).

1.2 The NAAA inhibitor AM9053 reduces tumour growth in the xenograft model of colon cancer

To assess the *in vivo* involvement of NAAA in murine carcinogenesis, we used AM9053, a selective NAAA inhibitor, in the xenograft model of colon cancer. CRC (HCT116) cells subcutaneously injected into immunodeficient mice gave rise to exponentially growing tumours. After 1 week, when the tumours became measurable, mice were treated (peritumorally, three times per week) with vehicle or AM9053 (20 mg/kg) until the sacrifice of the animals. AM9053 caused a significant reduction of xenograft tumour growth compared to vehicle-treated mice (Figure 24A). At the end of the pharmacological treatment (day 21), the tumour volume (mean ± SEM) for the vehicle and AM9053 groups were 1006.6±211 mm³ and 764.4±177 mm³, respectively. Also, we found a trend in reduction in the weight of tumours treated with AM9053 (Figure 24C).

1.3 Effect of the NAAA inhibitor AM9053 on NAAA and PEA targets expression in xenograft colon cancer tumours

In addition to PPAR-α, PEA can directly or indirectly activate GPR55, cannabinoids receptors and TRPV1 channel (Di Marzo et al., 2001, Ryberg et al., 20017, Petrosino and Di Marzo, 2017). Thus, we analyzed, by RT–PCR, the expression of such targets as well as of NAAA in xenografts tumours treated or not with AM9053. All the investigated targets were expressed in xenograft tumours, but the NAAA inhibitor AM9053 significantly reduced PPAR-α mRNA expression only (Figure 25).
Figure 23. Expression of NAAA and PPAR-α at different TNM stages of colorectal cancer (CRC) patients.

(A) Expression of hNAAA in colorectal cancer [data available in the web-accessible database GENT (Gene Expression across Normal and Tumour tissue)]. ***p<0.001 vs corresponding healthy tissues.

NAAA (B) and PPAR-α (C) mRNA expression was evaluated in colonic biopsies of patients at different stages (pT2, pT3 and pT4) of CRC. mRNA was evaluated by quantitative (real-time) RT-PCR analysis (mean ± SEM of 3 patients). *p<0.05 and **p<0.01 vs corresponding healthy tissues (i.e. adjacent non-tumour tissues)
Figure 24. Inhibitory effect of AM9053 (a NAAA inhibitor) on tumour formation in the xenograft model of colon cancer.
Tumours were induced by subcutaneous injection of colorectal cancer (HCT116) cells on the back of athymic female mice.
(A) Tumour growth was measured three times per week by a digital caliper, and tumour volume was calculated. AM9053 (20 mg/kg, peritumorally) treatment started 7 days after cell inoculation and it was administered three times per week for three weeks until the sacrifice of the animals.
(B) Representative images of mice after three weeks-treatment with vehicle or AM9053.
(C) Tumour weight calculated after the sacrifice of mice.
Results represent the mean ± SEM of 5 mice. *p < 0.05 vs vehicle-treated nude mice.
NAAA is crucially involved in proliferation, migration and in vivo colon tumorigenesis.

Figure 25. Expression of NAAA and PEA targets in the xenograft model of colon cancer
Human mRNA expression of NAAA (A), PPAR-α (B), TRPV1 (C), GPR55 (D), CB2 (E) and CB1 (F) was evaluated by quantitative (real-time) RT-PCR analysis (mean ± SEM of 5 mice) in xenograft tumour tissues. AM9053 (20 mg/kg, peritumorally) treatment started 7 days after cell inoculation and it was administered three times per week for three weeks until the sacrifice of the animals. *p < 0.05 vs vehicle-treated nude mice.
1.4 The incubation with xenograft tumour secretome reduces NAAA expression in colorectal cancer (CRC) cells

In preliminary experiments, we assessed NAAA mRNA expression in colorectal (HCT116 and Caco-2) cancer cells. Results showed the enzyme to be highly expressed in both cell lines (Table 5). Evidence from literature suggests that tumour secretome, consisting of factors derived from cancer stem cells, non-stem cells and stroma, plays a key role in cancer progression (Xue et al., 2008; Cordani et al., 2016). For this reason, we incubated the CRC (i.e. Caco-2) cells with conditioned medium derived from xenograft tumours and found that the tumour secretome reduced significantly NAAA (Figure 26).

1.5 The NAAA inhibitor AM9053 and its substrate PEA reduce the number of tumours induced by azoxymethane (AOM)

The AOM model of CRC was used to assess the in vivo chemopreventive potential of the NAAA inhibitor AM9053 and its substrate PEA. AOM treatment causes the formation of colonic tumours (Figure 27), which was significantly reduced by AM9053 (20 mg/kg, i.p.) or PEA (10 mg/kg, i.p.) (Figure 27). These results confirm a key role of NAAA and its substrate in experimental colon carcinogenesis.

AM9053 was well tolerated with control mice showing no weight loss or other signs of toxicity during treatment (data not shown)

1.6 AM9053 (NAAA inhibitor) and PEA inhibit the proliferation of colorectal cancer - but not of healthy colonic - cells

To evaluate the effect of AM9053 and PEA on cancer cell proliferation, we used the BrdU incorporation assay. We tested a number of non-cytotoxic concentrations of AM9053 (0.1-3 μM) and PEA (1-30 μM) on HCT116 cells. Both AM9053 and PEA significantly reduced the proliferation of colorectal cancer cells in a concentration-dependent manner after 24h of treatment (Figures 28A-B). Importantly, AM9053 at concentration of 3 μM inhibits NAAA and has no effect on FAAH activity, for which concentration of about 100 μM are needed (Bottemanne et al., 2018).

In order to evaluate the selectivity of AM9053 and PEA (tumoural vs non tumoural cells), the highest concentrations of both compounds (i.e. AM9053 3 μM and PEA 30 μM) were tested on healthy colonic epithelial cells (HCEC). Both compounds did not affect the proliferation of healthy cells, thus suggesting a selective antiproliferative effect vs tumoural cells (Figure 28C).

1.7 AM9053 and PEA treatments lessen the migration of colorectal cancer cells

To assess the effect of AM9053 (3 μM) and PEA (30 μM) on cell migration, a scratch assay was performed in two different cancer cell lines (i.e. Caco-2 and HCT116). Briefly, the capability of the epithelial cells to cover the scratch created by the insert is directly correlated to the cell migration.
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The distance between the edge of the cell sheet and the scratched line was measured. Both AM9053 (3 μM) and PEA (30 μM) (both 24-h treatment) significantly reduced the closure of wounds compared to vehicle-treated cells, as shown in the quantification of scratch expressed as percentage of healing in Figure 29 (top) and in the representative pictures in Figure 29 (bottom).
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**TABLE 5.** Detection of NAAA and GAPDH mRNA by quantitative (real-time) RT-PCR analysis in human colorectal (Caco-2 and HCT116) cells. (Threshold Cycle (Ct) ± S.E.M.)

<table>
<thead>
<tr>
<th>Target</th>
<th>Caco-2</th>
<th>HCT116</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAAA</td>
<td>25.53 ± 0.399</td>
<td>23.37 ± 0.079</td>
</tr>
<tr>
<td>GAPDH</td>
<td>17.60 ± 0.590</td>
<td>15.08 ± 0.138</td>
</tr>
</tbody>
</table>
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### Figure 26. Expression of NAAA in Caco-2 and HCT116 cells incubated with secretome

Caco-2 or HCT116 cells were cultured for 24h at 37°C in presence of secretome (i.e. conditioned medium derived from the tumour mass of the xenograft model of colon cancer). Control cells were incubated with normal medium. Human mRNA was evaluated by quantitative (real-time) RT-PCR analysis (mean ± SEM of 3 different experiments). ***p < 0.001 vs DMEM-treated Caco-2.
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**Figure 27.** Azoxymethane (AOM) model of colon cancer: chemopreventive effect of AM9053 (a NAAA inhibitor) and PEA. AM9053 (A, 20 mg/kg, i.p.) or PEA (B, 10 mg/kg, i.p.) were given three times a week for the whole duration of the experiment starting 1 week before the first administration of AOM. Measurements were performed 3 months after the first injection of AOM. Results represent the mean ± SEM of 9 mice. *p* < 0.05 and **p** < 0.01 vs AOM alone.
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Figure 28. Effect of AM9053 (a NAAA inhibitor) and PEA on colonic epithelial cell proliferation.

(A) AM9053 (0.1-3 μM) and (B) PEA (1-30 μM) were tested on proliferation of colorectal cancer (HCT116) cells by using the BrdU incorporation assay. Cells were incubated with increasing concentrations of compounds (24h-exposure). Each bar represents the mean ± SEM of three independent experiments. *$p < 0.05$ and ***$p < 0.001$ versus control (vehicle-treated cells).

(C) Effect of AM9053 (3 μM) or PEA (30 μM) on proliferation in healthy colonic epithelial cells (HCEC).
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**Figure 29. Effect of AM9053 (a NAAA inhibitor) and PEA on colorectal cancer cell migration**

Bottom: Effect of AM9053 (3 μM) and PEA (30 μM) in HCT116 (A) and in Caco-2 (B) monolayers after 24h treatment. The ordinates show the percentage of closure of the wound area. Each bar represents the mean ± SEM of three independent experiments. *p<0.05 and **P < 0.01 vs control (vehicle-treated cells).

Top: Representative pictures of CRC cells.
Chapter III. GPR35 promotes glycolysis, proliferation and colon tumorigenesis

1.1 Gpr35−/− BMDM have reduced glucose uptake

GPR35 is abundantly expressed in macrophages and in colonic epithelial cells (Wang et al., 2006). Macrophages exhibit different functional states, which can be appraised in vitro by differentiation under MΦ (baseline), M1 (‘inflammatory’, IFNγ+LPS) and M2 (‘regenerative’, IL-4) conditions (Murray et al., 2014). We have found that bone marrow derived macrophages (BMDM) from Gpr35−/− mice took up significant lower amounts of glucose compared to Gpr35+/+ macrophages (Figures 30A-C). These results were found in all phenotypes (MΦ, M1 and M2) of macrophages and were expressed as fmol of 2DG6P detected per cell after 10-60 min of 2DG incubation.

The reduced glucose uptake was not correlated with any difference in the expression of glucose transporters (i.e. GLUT1, SGLT1 and SGLT2) in MΦ, M1 and M2 macrophages between different genotypes (Gpr35+/+ vs Gpr35−/−) (Figure 31). As expected, we found a 4-fold increase in GLUT1 expression induced by LPS and IFNγ polarization in both wild type and Gpr35−/− M1 phenotype (Figure 31), confirming the importance of glucose as carbon source for pro-inflammatory macrophages. These data show that Gpr35−/− macrophages may need a lower amount of glycolysis substrate, suggesting the involvement of GPR35 in the cellular metabolism.

1.2 The lack of GPR35 impairs the metabolic profile of macrophages

To investigate whether reduced glucose uptake influenced glycolytic metabolism, we measured the extracellular acidification rate (ECAR), an indicator of glycolysis, in wild-type and knockdown M1 macrophages using the seahorse. Remarkably, metabolic study revealed that ECAR in Gpr35−/− M1 was significantly lower compared to wild type cells (Figure 31A). In fact, as shown in the Figure 31A, the knockdown cells had both reduced glycolysis and glycolytic capacity. Moreover, we proved that the lactate accumulation, a hallmark of increased glycolysis, is significantly reduced in Gpr35−/− M1 macrophages (Figure 31B).

Also, we assessed the metabolic profile of MΦ and M2 macrophages. Although inflammatory M1 macrophages support their energetic needs preferentially via aerobic glycolysis (Warburg effect), MΦ and M2 macrophages rely mostly on oxidative phosphorylation (OXPHOS) (Kelly and O'Neill, 2015). The oxygen consumption rate (OCR) was reduced in Gpr35−/− MΦ and Gpr35−/− M2 compared to wild-type cells which means that the lack of GPR35 impairs also the mitochondrial respiration in macrophages (Figures 31C-D). These data demonstrate that Gpr35−/− macrophages have a reduction in glycolysis and OXPHOS compared with wild type cells, suggesting that the receptor may play a pivotal role in energy production and cellular energetic demand.
Figure 30. Glucose uptake in in \textit{Gpr35}^{+/+} and \textit{Gpr35}^{-/-} bone marrow-derived macrophages (BMDM).
Rate of glucose uptake was measured in \textit{Gpr35}^{+/+} (red) and \textit{Gpr35}^{-/-} (blue) MΦ (A), M1 (B) and M2 (C) BMDM and was expressed as intracellular 2-deoxy-D-glucose-6-phosphate (2DG6P) concentration detected per cell after incubation of 2DG. *P < 0.05 and ***P < 0.001 vs wild-type cells.
Figure 31. Expression of glucose transporters in *Gpr35*+/+ and *Gpr35*−/− bone marrow-derived macrophages (BMDM).

Expression of GLUT1, SGLT1 and SGLT2 was evaluated in *Gpr35*+/+ (filled dots) and *Gpr35*−/− (empty dots) MΦ (black), M1 (blue) and M2 (red) BMDM. Murine mRNA was evaluated by quantitative (real-time) RT-PCR analysis (mean ± SEM of 3 mice). **P < 0.01 vs MΦ BMDM.
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Figure 32. Effect of GPR35 deletion on metabolic profile of macrophages.
(A) Extracellular acidification rate (ECAR) and (B) lactate production were measured in Gpr35<sup>+/+</sup>(red) and Gpr35<sup>-/-</sup> (blue) M1 macrophages. ECAR was measured during sequential injection of Glucose, Oligomycin and 2-deoxyglucose (2-DG). *P < 0.05 and **P < 0.001 vs wild-type BMDM (unpaired, Student's t-test).

The oxidative phosphorylation (OXPHOS) was measured in Gpr35<sup>+/+</sup>(red) and Gpr35<sup>-/-</sup> (blue) MΦ macrophages (C) and M2 macrophages (D), during sequential treatment with Oligomycin, FCCP and Rotenone/Antimycin. Data are from one experiment with three mice representative of three independent experiments. All data represented as mean ± SEM.
1.3 GPR35 influences angiogenesis via macrophages

Further, we explored the possible GPR35 function in the angiogenic process. CXCL1/GRO alpha, also known as KC in mouse, is a member of the CXC family of chemokines which promotes angiogenesis in colorectal cancer (Ohlsson et al., 2016). We measured the KC levels in supernatants of Gpr35+/+ and Gpr35−/− MΦ, M1 and M2 macrophages. M2-phenotype macrophages are mostly involved in promoting angiogenesis and contributing to tissue remodeling and tumour progression (Dong et al., 2017). As expected, we found a higher levels of KC in the supernatant of M2 compared with MΦ and M1 macrophages (Figure 33A). Interestingly, Gpr35−/− M2 macrophages produced a less amount of KC compared with wild type cells.

To investigate if macrophages may influence the formation of microvessel-like structures we assessed a co-culture system consisting of murine endothelial cell line (SVEC4-10) and M2-macrophages. SVEC cells were cultured for 6h at 37°C in presence of conditioned medium of wild type and knock-down M2 macrophages. Gpr35−/− M2- stimulated SVEC4-10 showed a trend towards reduction in all angiogenetic parameters (nodes, junctions, segments) compared to wild-type M2 macrophages cultured endothelial cells (Figure 33B for the representative images and for the quantification of tube network).

Finally, we studied the mechanism of angiogenesis in the aortic ring model. This model is based on the capacity of mouse aortic explants to form new vessels (Aplin et al. 2008). The system is regulated by macrophages, pericytes and fibroblast through a complex molecular cascade (Nicosia, 2009). Interestingly, when assessing sprout numbers, we observed a 65% reduction of ‘neo-vessels’ in aortas from mice lacking GPR35 (Figure 34). GPR35 was identified as a potential receptor for CXCL17, a mucosal chemokine that promotes the tumour growth and angiogenesis (Maravillas-Montero et al. 2017, Weinstein et al. 2006). We took advantage of pro-angiogenic effect of CXCL17 in the aortic ring model, treating both Gpr35+/+ and Gpr35−/− aortas. CXCL17 caused an increase (about 32%) of neo-vessels formation in Gpr35+/+ aortic rings, but not in KO aortas (Figure 34). These data confirm that the effect in aortic sprouts was mediated by GPR35.

All together these results show that the lack of GPR35 inhibits the formation of neovessels in vitro and ex vivo and interestingly the effect seems to be mediated by M2-macrophages.
GPR35 promotes glycolysis, proliferation and colon tumorigenesis

Figure 33. Effect of GPR35 deletion on angiogenesis via macrophages.
(A) CXCL1/GRO alpha, also known as KC in mouse is a member of the CXC family of chemokines. KC was detected in macrophages (MΦ, M1 and M2) supernatants.
(B) Representative images of SVEC cells incubated for 6h with conditioned media from Gpr35+/+ (M2 macrophages (left) and Gpr35−/− M2 macrophages (right).
(C) Quantification of tube network (i.e. number of junctions, number of segments and length of segments) was assessed using Image J with the Angiogenesis Analyzer in SVEC cells incubated for 6h with conditioned media from Gpr35+/+ M2 macrophages (red) and Gpr35−/− M2 macrophages (blue) or with RPMI (control, black). Data are from one experiment with three mice. All data represented as mean ± SEM.
Figure 34. The effect of lack of GPR35 in aortic ring model.
(bottom) Quantification of aortic sprouts from Gpr35<sup>+/+</sup> (red) and Gpr35<sup>-/-</sup> (blue) mice (left), treated or not with CXCL17 (0.2 mg/ml), was counted by using the microscope. Results represent the mean ± SEM of 5 mice *p<0.05.
(top) Representative image of aortic ring from wild-type mouse.
1.4 GPR35 is involved in experimental colitis-associated cancer and sporadic colon cancer

GPR35 is abundantly expressed in colonic epithelium; moreover, most cancer cells show conspicuous alterations in glucose metabolism. For this reason, we then evaluated whether GPR35 was involved in intestinal tumour progression using different models of intestinal tumour development.

In the CAC model, the number of colonic tumours significantly decreased in Gpr35−/− mice treated with AOM and DSS compared with wild type mice (Figure 35A), suggesting that the absence of GPR35 reduced the intestinal tumour growth driven by colitis. Also, the lack of GPR35 partially protected the mice from AOM/DSS-induced weight loss, compared with wild-type mice (Figure 35B).

Moreover, we also observed a significant decrease in number of intestinal tumours of 15-week-old Gpr35−/−;Apc<sup>min</sup> compared with Gpr35<sup>+/+</sup>;Apc<sup>min</sup> and Gpr35<sup>+/−</sup>;Apc<sup>min</sup> mice (Figure 35C). Therefore, the deletion of GPR35 protects mice from colitis-induced intestinal cancer and Apc<sup>Min</sup>-dependent intestinal tumorigenesis.

As this result was observed in global GPR35 knock-out mice we also have generated a conditional mouse lacking GPR35 in the intestinal epithelium (Gpr35<sup>ΔIEC</sup>). As shown in the Figure 35D Gpr35<sup>ΔIEC</sup>;Apc<sup>min</sup> mice had a significant lower amount of tumours compared with non-transgenic Apc<sup>min</sup> mice for GPR35 (Gpr35<sup>WT</sup>;Apc<sup>min</sup>), suggesting that the effect in tumour progression could be selectively mediated by GPR35 in the intestinal epithelial cells.

1.5 GPR35 affects intestinal epithelial cell turn-over under homeostatic conditions

Then, we investigated the role of GPR35 in the baseline turn-over of intestinal epithelial cells (IECs). As depicted in Figure 36, the number of IECs that had migrated along the crypt-villous axis after a 24h pulse of BrdU was reduced by more than 50% in Gpr35−/− compared to wild-type mice.

We subsequently investigated if GPR35 may act by directly promoting proliferation of the epithelial cells using colonic organoid cultures. This method allows long-term culture of isolated intestinal crypts or intestinal stem cells (Sato et al., 2009). The Figure 37 shows a representative image of colonic organoids from wild-type and GPR35-ko mice. Organoids from GPR35-ko mice had a significant lower number of Edu-positive cells compared with non-transgenic mice (Figure 37).

This means that the lack of GPR35 reduced the cell proliferation as indicated by EdU-incorporation in intestinal stem cells, located at crypt bottom of colonic organoids (Figure 37). These data support the finding that GPR35 promotes intestinal tumorigenesis through an increase of proliferation.
Figure 35. Role of GPR35 is involved in colitis-associated cancer and sporadic colon cancer.
(A) Number of tumours in the large intestine of 6-8 week-old Gpr35+/+ (red) and Gpr35−/− (blue) mice in the AOM-DSS model.
(B) Body weight comparison between 6-8 week-old Gpr35+/+ (red, n=19) and Gpr35−/− (blue, n=16) mice in the AOM-DSS model, normalized to day 0 within each cohort.
(C) Number of total tumours in the small and large intestine of 15-week-old Gpr35+/+, Apc^{min}, Gpr35+/−, Apc^{min} (grey) and Gpr35−/−, Apc^{min} (blue) littermates.
(D) Number of total tumours in the small and large intestine of 15-week-old Gpr35^{WT}, Apc^{min} (red, VC−/-) and Gpr35^{ΔIEC}, Apc^{min} (blue, VC+/-) littermates.
Figure 36. Role of GPR35 in the intestinal epithelial cell turnover under homeostatic conditions.
Immunohistochemistry of BrdU incorporation after a 24 hours pulse in intestinal sections from Gpr35<sup>+/+</sup> (red) and Gpr35<sup>−/−</sup> (blue) mice. Representative images (left panel). Quantification of cells staining positive for BrdU (right panel). Each data point represents one crypt villus axis from 3 individual mice per group.
Figure 37. Role of GPR35 in the epithelial cell proliferation using colonic organoid cultures.
(A) Representative images of colonic organoids grown out of colonic crypts collected from Gpr35+/+ (left) and Gpr35−/− (right) mice. Immunofluorescent staining of organoids was performed, and the cell proliferation was marked by incorporation of 5-ethynyl-2’-deoxyuridine (EdU, a thymidine analogue) into DNA.
(B) Quantification of number of EdU positive cells/organoid using Image J. All data represented as mean ± SEM. *P < 0.05 vs positive cells/organoid in wild-type mice.
DISCUSSION

Part I. Pharmacological inhibition of MAGL reduces experimental colon tumorigenesis

MAGL is a serine hydrolase that plays a major role in the degradation of the endocannabinoid 2-AG. MAGL and 2-AG have been involved in a number of tumour types (Nomura et al. 2010; Melck et al. 2000; Orellana-Serradell et al., 2015; Ligresti et al., 2003; Nithipatikom et al., 2011; Nomura et al., 2011). However, the role of MAGL in colorectal cancer physiopathology is both under-researched and controversial (Sun et al., 2013; Ye et al., 2011), with no study attempting to relate MAGL and its main substrate 2-AG to angiogenesis. Here, we have shown that MAGL and 2-AG are present in tumour tissues and that pharmacological inhibition of MAGL results in chemopreventive (AOM model) and curative (xenograft model) effects in colon carcinogenesis.

1.1 Presence of MAGL and 2-AG in the xenograft tumour tissue

Monoacylglycerols such as the endocannabinoid 2-AG, are metabolized to free fatty acids and glycerol by MAGL. MAGL has been shown to be up-regulated in aggressive compared to non-aggressive tumour cell lines as well as in primary tumours (Mulvihill and Nomura, 2013; Van Dross et al., 2013). Here, we have shown that tumour tissues generated by injection of human colorectal cancer (HCT116) cells in nude mice express both mRNA MAGL and its related protein. Due to the use of specific human antibodies, it is very likely that the detected MAGL mRNA in the tumour xenograft derives from human epithelial cells, possibly underwent to phenotypic changes during tumour implantation and growth. Others have shown that MAGL was up-regulated in colorectal cancer patient tissues (Ye et al., 2011).

Because MAGL is the main enzyme responsible for 2-AG degradation, we measured its levels in the tumour mass. 2-AG, which is known to exert antiproliferative effects in colorectal cancer cells (Ligresti et al., 2003) was shown to be elevated in the mouse colon with aberrant crypt foci induced by AOM (Izzo et al., 2008) as well as in colorectal polyps and tumours of CRC patients (Ligresti et al., 2003). In our study we have revealed, in tumour tissues, amounts (300 pmol/mg of lipid extract) of 2-AG that were about four-fold higher than those detected in HCT116 cells and healthy tissues derived from the contralateral flank of xenografted mice (i.e. the flank not injected with xenografted cells).

1.2 Effect of URB602 on 2-AG levels, tumour progression and angiogenesis

In order to evaluate tumour progression, angiogenesis and 2-AG levels associated to MAGL inhibition, we selected the MAGL inhibitor URB602, i.e. the first compound reported with selectivity for MAGL over fatty acid amide hydrolase (FAAH) (Fowler, 2012). Although the in vitro selectivity of high concentrations of this compound (2-AG vs anandamide hydrolysis) has been questioned (Vandevoorde et al., 2007), its functional selectivity has been demonstrated by the
Discussions - Part I

Pharmacological inhibition of MA
GL reduces experimental colon tumorigenesis

ability to elevate 2-AG levels in hippocampal slice cultures without affecting levels of other endocannabinoid-related molecules (Hohmann, et al., 2005; King et al., 2007). In contrast to directly-activating CB1 receptor agonists, URB602, given i.p. at doses less than 10 mg/kg, did not induce catalepsy and hypothermia (Comelli et al., 2007).

i) Effect of URB602 on 2-AG levels in xenograft tumour tissues.

We have found that URB602, at the daily dose of 5 mg/kg, which is significantly lower than that found to be active at, e.g., reducing upper intestinal transit (Duncan et al., 2008), showed only a non-significant trend toward an increase in 2-AG levels in the xenograft tumour tissue. The failure of URB602 to significantly increase 2-AG levels could be due to the high levels of the endocannabinoid already present in the tumour tissue (see above). In fact, under the same experimental conditions, URB602 increased 2-AG levels in healthy tissues, where the basal levels of 2-AG are not so high like in tumour mass. Importantly, in this set of experiments, we found that URB602 significantly and selectively increased (by approximately four-fold) 2-AG levels, without significantly affecting the levels of anandamide (non-significant two-fold increase) and the related acylethanolamides (i.e. PEA and OEA).

ii) Effect of URB602 on xenograft tumour progression

Previous studies have shown that 2-AG as well as inhibitors of 2-AG hydrolysis exerted antitumorigenic (antiproliferative, inhibition of invasion and cell migration) effects in a number of cancer cell lines, including prostate, ovarian, breast and melanoma cells (Mulvihill and Nomura, 2013; Van Dross et al., 2013). However, the role of MAGL in colorectal cancer cells is controversial. Ye and colleagues found that colorectal cancer cell growth and invasion were inhibited by pharmacological and siRNA mediated MAGL knock-down (Ye et al., 2011). By contrast, a more recent paper highlighted the potential tumour suppressive role of MAGL in colorectal cancer cell lines, based on the findings that over-expression of MAGL suppressed colony formation in cell lines and knockdown of MAGL resulted in increased Akt phosphorylation (Sun et al., 2013). In the present study we have shown that URB602, at the daily dose of 5 mg/kg, reduced the growth of tumours generated by xenograft injection of colorectal cancer cells in athymic mice. It is important to further emphasize that URB602 reduced tumour growth at a dose devoid of central side effects (Comelli et al., 2007) and which selectively inhibits the metabolism of 2-AG without affecting the hydrolysis of anandamide and related acylethanolamide (present results, see above). Consistent with our data, Ye and colleagues showed that JZL184, a potent and selective MAGL inhibitor, reduced tumour formation generated by xenograft injection of colorectal cancer (Caco-2) cells in nude mice (Ye et al., 2011).
iii) Effect of URB602 on angiogenesis and proliferation in xenograft tumour tissues

A fundamental feature of tumour progression is the accomplishment of abundant vascular development to sustain the growing tumour mass (Hanahan and Folkman, 1996). Therefore, we evaluated the impact of URB602 on a number of angiogenesis markers in tumour-bearing mice. Although no specific study on 2-AG or MAGL on angiogenesis exists, cannabinoids, in addition to their cancer cell death promoting effects, have been also shown to normalize tumour vasculature (Blázquez et al., 2003; Pisanti et al., 2011; Pisanti et al., 2013; Velasco et al., 2015). In cancer cells, cannabinoids inhibit the activation of the vascular endothelial growth factor (VEGF) cascade, which is considered the most important and well-characterized pathway contributing to angiogenesis (Ferrara, 2004; Shibuya, 2011), and a number of elements of this cascade are down-regulated by cannabinoids, for example, in skin carcinomas, gliomas and thyroid carcinomas [see Velasco et al., 2016, for review]. Here, we have provided a number of evidences suggesting that URB602 acts via inhibition of angiogenesis. Indeed: URB602 i) reduced the expression of VEGF, which is considered the most important and the well-characterized contributor to angiogenesis (Ferrara, 2004; Shibuya, 2011); ii) reduced density, structure and composition of tumour vessels. Furthermore, using HUVEC we were able to recapitulate in vitro the anti-angiogenic effect of URB602. To the best of our knowledge, this represents the first demonstration about the involvement of MAGL and of its main substrate 2-AG in angiogenesis.

Lastly, our results show that the beneficial effect of URB602 was associated to down-regulation of cyclin D1, a specific cyclin required for tumour progression. Down-regulation of cyclin D1 is known to restrict the cell cycle progression to the G0/G1 phase. Conversely, cyclin D1 over-expression has been observed in 68.3% of human colorectal cancer, suggesting a role in human colorectal tumorigenesis (Bahnassy et al., 2004). Others have shown that the putative endocannabinoid noladin ether down-regulated cyclin D1 expression in prostate cancer cells and that Met-F-anandamide (a synthetic analogue of anandamide) in combination with the FAAH inhibitor URB597, induces G0/G1 cell cycle arrest by down-regulating cyclin D1 (Ravi et al., 2014). However, in the present study no significant effect of URB602 on the cycle-negative regulator p27KIP was observed.

1.3 Effect of URB602 in the azoxymethane (AOM) model of colon carcinogenesis

In a different set of experiments, we evaluated the chemopreventive effect of URB602 in a model of colon carcinogenesis induced by AOM. Because the development of colon cancer spans about 10–15 years, chemoprevention is a great opportunity to counteract this fatal disease. The AOM model can experimentally induce carcinogenesis similar to the pathogenesis of human sporadic colon
cancer. In support to this assertion, there is the observation that sporadic CRC and the AOM model share several pathways and mechanisms, including K-ras, β-catenin and TGFβ mutations. (Chen and Huang, 2009). Repetitive intra-peritoneal treatment of mice with AOM causes the formation of preneoplastic lesions (ACF), polyps and tumours in the distal colon. We have previously shown that the pharmacological enhancement of endocannabinoid levels (through inhibition of anandamide hydrolysis by the FAAH inhibitor N-arachidonoylserotonin) as well as the cannabinoid receptor agonist HU-210 attenuated the development of ACF in the mouse colon (Izzo et al., 2008). Here, we have shown that MAGL is over-expressed in the colon of AOM-treated mice and, more importantly, that URB602 reduced, approximately by one half, the number of ACF, polyps and tumours. Importantly, the effect of URB602 was absent in MAGL-deficient mice, thus suggesting that the drug is acting selectively via inhibition of MAGL and not through other potential unspecific mechanisms.

1.4 Concluding remarks

In summary, we have shown that MAGL and its main substrate 2-AG are abundantly present in the colorectal cancer tissues and that a pharmacological inhibition of the enzyme by URB602, which selectively increases 2-AG in vivo, resulted in chemopreventive and curative effects in experimental models of colon carcinogenesis. Importantly, the chemopreventive effect of URB602 was abrogated in Mgl1-null mice. The antitumour effect of URB602 in vivo was associated to inhibition of angiogenic process and reduction of cyclin D1, a marker of cell proliferation. Furthermore, studies on human endothelial cells have shown that the URB602 exerted a direct antiangiogenic effect.
Discussion - Part III

Part II. NAAA is crucially involved in proliferation, migration and \textit{in vivo} colon tumorigenesis

NAAA is a lysosomal enzyme which hydrolyses a number of N-acylethanolamines at acidic pH. The preferred NAAA substrate is PEA, a lipid mediator chemically-related to the endocannabinoid anandamide.

Although NAAA role has been investigated in bladder and prostate cancer (Wang et al., 2008; Liu et al., 2014; Du et al., 2016; Vago et al., 2017), no information has been published to date about its possible physiopathological role in CRC. Here, we have shown that i) NAAA is down-regulated in human colorectal cancer biopsies and ii) pharmacological inhibition of NAAA – as well as PEA administration - reduces experimental colon carcinogenesis \textit{in vivo} and decreases the proliferation and migration of human colorectal cancer cell lines.

\subsection*{1.1 NAAA and PPAR–α expression in human CRC}

NAAA expression has been shown to be higher in non-aggressive prostate cancer (PC) - compared with aggressive PC - (Liu et al., 2014) and to be up-regulated in aggressive mouse ovarian cancer (Du et al., 2016). We observed a significant reduction in NAAA expression in CRC tissues compared to that detected in adjacent non-tumours tissues. Because NAAA is the main enzyme responsible of PEA degradation (Alhouayek et al., 2015), we next focused on the expression of the key targets of PEA in different TNM stages of CRC patients. PEA is a bioactive lipid that strongly alleviates pain and inflammation in animal models and in humans. Several studies demonstrated that PEA can act via direct activation of the PPAR-α (Lo Verme et al., 2005) and many of its pharmacological properties are generally mediated by PPAR-α (Alhouayek and Muccioli, 2014; Gugliandolo et al., 2018; Esposito and Cuzzocrea, 2013; Skaper et al., 2015; Iannotti et al., 2016). Here, we have found that PPAR-α was down-regulated in tumours of patients with stage pT3 and pT4 compared to normal tissues. PPAR-α function in cancer is still uncertain and currently under investigation, and it may be differently related to cancer/cell type of the tumour (Gou et al., 2017). Both PPAR-α agonists and antagonists have been shown to exert antiproliferative effects in cancer cells and to attenuate carcinogenesis \textit{in vivo}. For example, PPAR-α activation increases proliferation in breast and renal cancer cell lines (Suchanek et al., 2002; Abu Aboud et al., 2013) and causes liver cancer in rodents, with PPAR-α null-mice to be resistant to hepatocarcinogenic effects of PPAR-α agonists (Peters et al., 1997; Zak et al., 2010; Li et al., 2014; Hann et al., 2013). On the other hand, the PPAR-α antagonists MK886 and NXT629 also inhibit chronic lymphocytic leukemia (CLL) cell proliferation (Spaner et al., 2013; Messmer et al., 2015).

\subsection*{1.2 Effect of AM9053 on experimental carcinogenesis}

In order to assess the role of NAAA on experimental tumour progression, we took advantage of AM9053, a potent and selective NAAA inhibitor. Although its electrophilic isothiocyanate moiety,
AM9053 reversibly interacts with the enzyme (West et al., 2012; Malamas et al., 2015). AM9053 is the first NAAA inhibitor active following systemic administration *in vivo* (Alhouayek et al., 2015) and its selectivity has been demonstrated by the ability to increase PEA, but not AEA, levels in experimental colitis (Alhouayek et al., 2015).

*i) Effect of AM9053 on xenograft tumour growth.*

In the present study, we have demonstrated that AM9053, at dose of 20 mg/kg, reduced the xenograft tumour growth generated by injection of colorectal cancer cells (HCT116) in athymic mice. In line with our results, Alhouayek and colleagues showed that AM9053, at same dose, reduced colonic inflammation, an effect associated with a selective increase of endogenous PEA levels (Alhouayek et al., 2015). This observation is relevant in the well-established relationship existing between intestinal inflammation and CRC genesis and development (Terzic et al., 2010). Others have been shown that NAAA inhibitors are effective in experimental models of inflammation and pain (Bonezzi et al., 2016; Petrosino et al., 2015; Sasso et al., 2018; Alhouayek et al., 2015).

To further explore AM9053 mode of action, we considered the possible involvement of receptors (i.e. CB receptors, TRPV1, GPR55 and PPAR-α), which have been shown, based on pharmacodynamic studies, to be targeted by PEA (Petrosino and Di Marzo, 2017). Among PEA targets, we have found a reduction of PPAR-α expression only, following AM9053 treatment, suggesting a possible involvement of such target in the antitumoural effect of AM9053.

**ii) Effect of AM9053 and exogenous PEA on tumours induced by azoxymethane (AOM)**

We provided further evidence to support the role of NAAA in the experimental colon carcinogenesis by using the AOM model of colon cancer (Neufert et al., 2007). AOM is a potent carcinogen that cause a high number of tumours in the distal colon. This model is extensively used to study the mechanisms underlying human sporadic colon cancer as well as to evaluate drug potential chemopreventive effects (Neufert et al., 2007).

Previous studies have shown that pharmacological inhibition of FAAH and MAGL, by increasing the levels of AEA and 2-AG, respectively, reduced the development of tumours in the AOM model (Izzo et al., 2008, Pagano et al., 2017 see Chapter I). Here, we have shown that AM9053 reduced, approximately by 40%, the number of AOM-induced tumours. Similar to NAAA inhibition, exogenous administration of PEA produced a significant reduction in the tumours number caused by the injection of AOM.
1.3 Effect of AM9053 and exogenous PEA on proliferation and migration of colorectal cancer cells

We have here reported, for the first time, that NAAA mRNA is expressed in two different colorectal cancer cell lines, i.e. Caco-2 and HCT116. More importantly, we have found a down-regulation of NAAA in Caco-2 and HCT116 cells incubated with secretome derived from xenograft tumours. In recent years, the innovative proteomic technologies have greatly accelerated studies on the cancer secretome (Xue et al., 2008; Hsiao et al., 2017). Tumoural and stromal cells secrete a number of proteins, including growth factors, proteinase, immunoregulatory cytokines, cell motility factors or other bioactive molecules able to crucially regulate the tumour microenvironment (Hsiao et al., 2017; Cordani et al., 2016). Indeed, these cancer secreted proteins are essential in the processes of differentiation, invasion, metastasis and angiogenesis by regulating cell-to-cell and cell-to-extracellular matrix interaction (Xue et al., 2008; Cordani et al., 2016). The effect of cancer secretome (from xenograft model) on NAAA expression in Caco-2 cells adds further support to the observation that NAAA is dysregulated in colon cancer patients and that the tumoural microenvironment may be responsible of such change in gene expression.

The antitumoral effects of the NAAA inhibitor AM9053 and of exogenous PEA were also depicted by the decreased proliferation and migration of CRC cells. Indeed, we have shown that, in a concentration-dependent manner, AM9053 and PEA reduced proliferation in two colorectal carcinoma cell lines, i.e. Caco-2 and HCT 116 cells. It is noteworthy that the antiproliferative effect of AM9053 was observed at concentrations which were able to inhibit NAAA, but not FAAH, in enzyme assays (Malamas et al., 2015).

Importantly from a translational viewpoint, the effect of both AM9053 and PEA was specific for colorectal carcinoma cells, showing no antiproliferative effects in healthy colonic epithelial cells. Others have shown that PEA caused a reduction in proliferation and VEGF signalling in Caco-2 cells through a selective PPAR-α-dependent inhibition of Akt/mTOR pathway (Sarnelli et al., 2016).

It is well established that tumour metastasis, i.e. the movement of tumour cells from a primary site, is the major responsible for the deaths of cancer patients and that cell migration is the first step in the metastatic process (Wirtz et al., 2011; Steeg, 2016). Therefore, we evaluated the impact of AM9053 and PEA on the capability of CRC cells to migrate. By using the scratch assay, a method that measures cell migration in vitro, we have here demonstrated that pharmacological inhibition of NAAA, which is expected to increase PEA levels, or exogenous administration of PEA reduced the migration of CRC cells.
1.4 Concluding remarks

In summary, we put forth the role of NAAA in colorectal cancer and propose NAAA inhibitors as a potential therapeutic strategy in CRC. Specifically, we have shown that NAAA inhibition results in antitumoural actions in two experimental models of colon cancer, i.e. the xenograft model and the model of chemoprevention induced by the carcinogenic agent AOM. Furthermore, studies on cells have shown that the NAAA inhibitor AM9053 reduced cell migration and exerted antiproliferative effects in tumoural – but not healthy – cells. Further studies using the genetic silencing of the enzyme will help us to confirm the role of NAAA in colon cancer development.
Part III. GPR35 promotes glycolysis, proliferation and colon tumorigenesis

GPR35, an orphan G protein coupled receptor, is mainly expressed in the colon and immune system (Wang et al., 2006). A GWAS identified the \textit{GPR35\textsuperscript{T108M}} polymorphism as novel risk locus for UC and PSC, that are diseases with a high-risk developing cancer (Ellinghaus et al. 2013). GPR35 seems to be upregulated in breast, gastric and non-small-cell lung cancer tissues, although its role and the signalling pathways are unknown (Guo et al., 2017; Okumura et al., 2004). Here, we have shown that GPR35 deletion in mice results in a decrease of i) glucose metabolism in macrophages, ii) colonic epithelium turnover and iii) number of tumours in experimental models of colon cancer.

1.1 GPR35 controls macrophages metabolism

Macrophages display a spectrum of functional activation phenotypes depending on the composition of microenvironment [\textit{e.g.} variations in concentrations of metabolites (O’Neill, 2011; Tannahill et al., 2013; Rodriguez-Prados et al., 2010), lipids (El Kasmi et al., 2013), oxygen tension (Kelly and O’Neill, 2015), and cytokines (Hashimoto-Kataoka et al., 2015; Idzko et al., 2014)] (El Kasmi and Stenmark, 2015). Despite this huge range of \textit{stimuli}, two distinct macrophage activation states (also called ‘canonical activation’) have been recognized, namely the M1 (or classically activated) macrophages and the M2 (or alternative activated) macrophages (Biswas and Mantovani, 2010). Polarized macrophages show also a distinct regulation of glucose metabolism, thus M1 phenotype displays a metabolic shift toward the anaerobic glycolytic pathway (Kelly and O’Neill, 2015).

In order to explore the GPR35 role in cell metabolism, we used the canonical activation of macrophages towards M1 and M2. We found that glucose uptake was deeply reduced in \(Gpr35^{/-}\) BMDM, irrespective of polarizing conditions. Importantly, the reduced glucose uptake was not due to a different expression of glucose transporters, although a predominant role of GLUT1 was revealed functionally. GLUT1 mediates the constitutive glucose uptake, which occurs by facilitated diffusion in most cells and tissues (Lee et al., 2015) This suggests that the amount of consumed glucose drives the uptake. Therefore, \(Gpr35^{/-}\) macrophages may need a lower amount of glycolysis substrate, suggesting an involvement of GPR35 in the cell energetic demand.

Thereafter, we assessed the metabolic profile of \(\Phi\), M1 and M2 BMDM. The increased glycolysis in M1-polarized macrophages is permissive to quickly trigger microbicidal activity and cope with a hypoxic tissue microenvironment (Biswas and Mantovani, 2013). Interestingly, we proved that the lack of GPR35 resulted in a reduction in glycolysis and glycolytic capacity (as showed by extracellular ratification rate) in M1 BMDM compared to \(Gpr35^{+/+}\) BMDM, with a consequent decrease in lactic acid, the final product of aerobic glycolysis.

Although inflammatory M1 macrophages support their energetic needs preferentially via aerobic glycolysis (Warburg effect), \(\Phi\) and M2 macrophages rely mostly on oxidative phosphorylation
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Additionally, here we have shown that oxygen consumption rate (OCR), a measure of OXPHOS was also reduced in Gpr35−/− MΦ and M2 BMDM compared to wild-type cells, thus confirming that GPR35 is a key actor in cell energetic demand and production.

1.2 GPR35 affects angiogenesis

M2-polarized macrophages are known to contribute to tissue remodeling, repair, and wound healing under pathophysiological conditions (Biswas and Mantovani, 2010). Also, M2 macrophages may influence angiogenesis via both direct and indirect mechanisms, including elevated production of key angiogenic factors and secretion of matrix metalloproteinases, thus facilitating endothelial cell migration and tube formation (Zajac et al., 2013).

To verify if GPR35 interferes in the pro-angiogenic role of M2-macrophages, we used a co-culture system consisting of murine endothelial cells and M2-macrophages. Endothelial cells showed a reduction in the formation of microvessel-like structures when incubated with conditioned medium of Gpr35−/− M2 BMDM. Furthermore, we have showed that Gpr35−/− M2 BMDM produced lower amounts CXCL1 compared to wild type cells, which could explain the effect of conditioned medium on endothelial cells first mentioned. CXCL1 is the most significant cytokines derived from tumour-associated macrophages for inducing breast cancer metastasis (Wang et al., 2018). Also, CXCL1 high levels promote tumorigenicity and serve as a poor prognostic biomarker in metastatic CRC patients (le Rolle et al., 2015).

Finally, we observed that the capacity of the aortic endothelium to form neo-vessels was reduced (about 65%) in Gpr35−/− mice. The aortic ring model is one of the most widely used methods to study angiogenesis and its mechanism, including the regulation of angiogenesis by macrophages, pericytes and fibroblasts (Aplin et al., 2008). It is worthy to note that CXCL17, i.e. a supposed GPR35 ligand, increased the neo-vessels formation only in the aortic cultures of wild type mice, but not in Gpr35−/− aortic endothelium.

In conclusion, we provided evidence that the lack of GPR35 reduced neo-vessels formation, with M2 macrophages playing a crucial role.

1.3 GPR35 has a role in colonic proliferation and tumorigenesis

Because GPR35 is highly expressed in the intestine, we investigated the possible role of GPR35 in the intestinal epithelial cells (IECs). The number of IECs that migrated along the crypt-villous axis after a 24h treatment of BrdU was reduced by more than 50% in Gpr35−/− compared to wild-type mice. Similarly, colonic organoids derived from Gpr35−/− mice exhibited decreased proliferation as determined by fewer cells incorporating EdU compared to cultures derived from wild-type mice. These data demonstrated a ~40% reduction in the turn-over of the intestinal epithelium in the
absence of GPR35. The 3D organoid model is the most useful and innovative long-term culture of primary murine colonic epithelial cells mimicking intestinal epithelial function (Sato et al. 2009). Finally, we observed a protective effect of GPR35 deletion, resulting in a reduction of tumours, in the Apc\textsuperscript{\textit{min}} model of CRC sporadic and in the AOM/DSS model of CAC. Additionally, in the Apc\textsuperscript{\textit{min}} model we have demonstrated that the protective effect was selectively mediated by GPR35. Indeed, Gpr35\textsuperscript{AIEC} (conditional mice lacking GPR35 in the intestinal epithelium); Apc\textsuperscript{\textit{min}} mice had a significant lower number of tumours compared to non-transgenic Apc\textsuperscript{\textit{min}} mice for GPR35 (Gpr35\textsuperscript{WT}; Apc\textsuperscript{\textit{min}}).

### 1.4 Concluding remarks

We have shown that GPR35 plays a key role in cell energetic demand, as revealed in metabolic studies on macrophages. Moreover, the deletion of GPR35 in M2-macrophages resulted in a reduced ability to produce pro-angiogenic factors. Finally, GPR35 regulated the formation of neovessels in the aortic epithelium.

In vivo, the deletion of GPR35 reduced the turnover of intestinal epithelial cells in physiological conditions and protected mice from tumours formation in two different models of colon cancer. The effect on colon tumorigenesis could be due to GPR35 impact on cell metabolic profile, since it has been demonstrated that cancer cells rewire their metabolism to promote growth, survival, proliferation, and long-term maintenance.
GENERAL CONCLUSIONS

Colorectal cancer (CRC) is the most common gastrointestinal malignancy. Despite the continuous progresses in CRC diagnostic and therapeutic systems, which have reduced recurrence rates and are prolonging survival, steps forward can be achieved only by pointing to a better understanding of its pathogenesis.

The experiments depicted in this PhD thesis highlight three potential targets, i.e. MAGL, NAAA and GPR35, implicated in colon carcinogenesis (see graphical conclusion).

Briefly, we have shown that:

- MAGL inhibition with URB602 was effective at increasing the endogenous levels of the endocannabinoid 2-AG and resulted in antitumour effects in vivo. The pharmacological inhibition of URB602 was selective for MAGL as demonstrated in Mgl-null mice. More in depth studies have demonstrated that tumour growth reduction may due to both proliferation and angiogenesis inhibition.

- NAAA was down-regulated in human and mouse colon cancer tissues. Both NAAA inhibition, by AM9053, and exogenous administration of PEA (i.e. the NAAA main physiological substrate) caused a reduction of colon cancer growth in vivo. AM9053 and PEA were effective antiproliferative agents, showing a selectivity toward CRC vs healthy colonic cells. It is worthy to emphasise that PEA is safe in humans and it is commercially available – as foods for special medical purposes – to alleviate inflammatory and painful conditions.

- GPR35 deletion impaired cell energetic demand in macrophages and reduced their ability to stimulate neo-vessels formation in the endothelium. Also, GPR35 physiologically regulated the turnover of intestinal epithelial cells in both in vivo and ex vivo models. Finally, GPR35 deletion protected mice from tumour formation in two different models of colon carcinogenesis. Notably, inactivation of conditional GPR35 by villin-cre, which results in a selective GPR35 deletion in the intestine, caused less tumours development in APCmin mice.

In conclusion, this PhD thesis contributes to elucidate the physiopathological role of MAGL, NAAA and GPR35 in colon carcinogenesis and thus may offer novel future pharmacological opportunities for colon cancer prevention and cure.
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