EFFECT OF SUB-TOXIC CONCENTRATIONS OF MEAT METABOLITES ON COLON CANCER CELL GROWTH AND OXIDATIVE STRESS

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Ghent, June 2017

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Acknowledgements

Five years ago, I set foot on the campus Coupure of the bio-engineers for the first time, nervous, curious, not knowing what to expect. A lot of hard work, stress, fun and unforgettable days and nights later, the end of this amazing period is near. An infinite amount of fantastic memories were made, that I will carry on in my heart forever. The past year has been a year of 'last times', but also the year of the master thesis. Right from the start that I came into contact with food science, I knew that I wanted to do my thesis within this field. I am very grateful for the opportunities that I got. The whole process of working on this thesis has been an enriching experience, and I want to thank everybody who played a role in making this possible.

First of all I want to express my gratitude to Prof. dr. ir. John Van Camp and Prof. dr. ir. Stefaan De Smet for the opportunity to work on their project and for the support during the process. The monthly discussions and suggestions have been extremely useful in the process of completing this thesis. Thank you for the great tips and critical reviews along the road, and for the trust you gave me.

A special thanks to my supervisor dr. ir. Charlotte Grootaert, for her never-ending enthusiasm and positivism in the lab, for teaching me to work independently and to think critically, for supporting me when my mind was clouded, for giving constructive feedback, and to push me into the right direction when necessary. I could not have wished for a better tutor!

I would also like to thank Prof. dr. ir. Olivier De Wever and dr. ir. Glenn Wagemans for the interesting meetings and the opportunity to work in their lab. Thanks to this, new perspectives arose that added a lot of value to this work.

In addition, I want to thank the whole lab group for the help during my lab work. I enjoyed working with all of you.

Finally, many thanks go to my friends and family, for supporting me through the ups and downs. I know that I can be a handful once in a while, and I'm blessed to have them around. Thanks mom and dad for the numerous pep talks and for believing in me, thanks little bro to make me laugh even when I don't feel like smiling at all, you mean the world to me.

Abstract

Red and processed meat have been correlated with colon cancer development, and are therefore classified as Group 2A and 1 carcinogens, respectively. Although many studies investigated the mechanism behind this effect, most studies have focused on the initial stages of colon cancer development, i.e. mutations in the intestinal cells, rather than on further stages of colon cancer progression, i.e. proliferation, metastasis and invasion, and also the long-term impact of the micro-environment, as defined by red/processed meat containing meals, is largely unexplored.

In this work, a novel long-term approach was used to investigate the impact of sub-toxic exposure of three different intestinal cancer cell lines (Caco-2, HT-29 and HCT 116) to meat matrix components and microbial metabolites formed after the consumption of (processed) red meat, including nitrite, hemoglobin, butyrate, hemin and kynurenine. The sub-toxic concentration (MTT, SRB and LDH assay) and short-term effects were determined by a three day exposure with the selected components. Next, the cells were exposed to sub-toxic concentrations for up to twelve days. Effects on oxidative stress (GSH/GSSG ratio) and cell proliferation (MTT and SRB assay, IncuCyte Live Cell Analysis), rather than on cell adhesion (clonogenic assay), were observed, and these effects were compound and cell line specific.

Nitrite and hemoglobin did not influence the cell parameters in any way, thereby suggesting an indirect effect of nitrite on carcinogenesis. Hemin served as an alternative heme-containing compound, and exposure to concentrations of 0.01 up to 10 µM gave rise to proliferation in Caco-2 cells but not HCT 116 cells. Sodium butyrate most likely induced differentiation and cell cycle arrest when added in concentrations ranging between 0.5 and 12.5 mM, whereas improved cell growth was observed for long-term exposure to much lower levels of butyrate, being 1-50 µM. Kynurenine, a primary metabolite of the tryptophan depletion pathway, resulted in higher cellular activity and cell numbers upon long-term treatment. The XF24 Seahorse technology revealed that in long-term pretreated HT-29 cells, shifts from aerobic to anaerobic metabolism - the Warburg effect, characteristic for cancerous phenotypes - occurred after hemin treatment, and for kynurenine treatment, the aerobic mitochondrial respiration was disturbed as well.

In conclusion, with the novel *in vitro* long-term approach we have opened perspectives that contribute in unravelling the complex relation between the consumption of red meat and the higher risk of colorectal cancer, especially in the later stages of colon cancer development.

Keywords: Red meat, colorectal cancer, meat components, microbial metabolites, long-term exposure

Samenvatting

Rood en verwerkt vlees werden reeds gecorreleerd met colonkanker ontwikkeling, en worden daarom respectievelijk geclassificeerd als Groep 2A en 1 carcinogenen. Veel studies hebben zich toegelegd op het ontrafelen van de mechanismen achter dit effect, maar de focus lag daarbij vooral op de initiële stadia van colonkankerontwikkeling, met name mutaties in de intestinale cellen, eerder dan op latere stadia van colonkanker progressie, met name proliferatie, metastase en invasie. Bovendien is ook de lange termijn impact van de micro-omgeving van tumorcellen, zoals bepaald door rood/verwerkt vlees consumptie, grotendeels onbekend.

In deze masterthesis werd een nieuwe lange termijn set up gebruikt om drie cellijnen van darmkankeroorsprong (Caco-2, HT-29 en HCT 116) bloot te stellen aan sub-toxische concentraties van componenten in vlees en microbiële metabolieten die ontstaan na de consumptie van (verwerkt) rood vlees, zijnde nitriet, hemoglobine, butyraat, hemine en kynurenine. De sub-toxische concentratie range (MTT, SRB en LDH assay) en effecten op korte termijn werden bepaald door een driedaagse blootstelling aan de gekozen componenten. Vervolgens werden de cellen blootgesteld aan sub-toxische concentraties voor een periode tot 12 dagen. We observeerden effecten op oxidatieve stress (GSH/GSSG) en cellulaire proliferatie (MTT en SRB assay, IncuCyte Live Cell Analysis), eerder dan op celaanhechting (clonogenic assay), en deze effecten waren component- en cellijnspecifiek.

Nitriet en hemoglobine hadden geen invloed op de cellulaire parameters, waardoor bevestigd wordt dat nitriet, een conserveermiddel dat vaak in vlees toegevoegd wordt, een indirect effect heeft op kankerontwikkeling. Hemine werd gebruikt als alternatieve heem-bevattende component, en had in concentraties van 0.01 tot 10 µM een duidelijk prolifererend effect of Caco-2 cellen, maar niet op HCT 116 cellen. Natriumbutyraat induceerde een differentiatie en een groeistop bij concentraties tussen 0.5 en 12.5 mM, terwijl juist groeistimulatie geobserveerd werd bij lange termijnblootstelling aan veel lagere butyraat gehaltes, zijnde 1-50 µM. Kynurenine is een primair metaboliet in de afbraakreactie van tryptofaan, en blootstelling van de cellen aan deze component voor langere tijd resulteerde in een hogere activiteit van de cellen en een groter celaantal. De XF24 Seahorse technologie toonde dat HT-29 cellen na lange termijn blootstelling van hemine een shift vertonen van aeroob naar anaeroob metabolisme - het Warburg effect, wat karakteristiek is voor kankerfenotypes - en dat kynurenine behandeling de aerobe mitochondriale respiratie ook verstoorde.

Samenvattend werden nieuwe perspectieven bekomen via deze nieuwe *in vitro* lange termijn techniek, die een goede aanvulling kunnen zijn op de huidige kennis rond rood vlees en het risico op colonkanker,

en kunnen bijdragen tot het ontrafelen van de complexe relatie tussen deze twee factoren, in het bijzonder voor de latere stadia van colonkanker ontwikkeling.

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List of abbreviations

ACF	Aberrant crypt foci
AHR	Aryl hydrocarbon receptor
APC	Adenomatous polyposis coli
CRC	Colorectal cancer
CSC	Cancer stem cell
DMEM	Dulbecco's Modified Eagle's Medium
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-mesenchymal transition
FCCP	carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone
GPCR	G-protein-coupled receptor
GSH	Glutathione
GSSG	Glutathione disulfide
IARC	International Agency for Research on Cancer
IDO	Indoleamine 2,3-dioxygenase
LDH	Lactate dehydrogenase
NCD	Non-communicable disease
NEM	N-Ethylmaleimide
NOC	N-nitroso compound
OCR	Oxygen consumption rate
OPT	O-phtalaldehyde
SCFA	Short chain fatty acids
TDO	Tryptophan dioxygenase
UN	The United Nations
WCRF	World Cancer Research Fund
WHO	World Health Organization

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Problem statement

Nowadays, the trend of 'a healthy lifestyle' is booming. Consumers are more and more interested in what they eat and are becoming more critical with regard to the health and safety aspects of foods. One of the most discussed food products these days is meat, especially red meat and processed meat. Red meat includes beef, lamb, veal and pork, whereas white meat includes chicken, game and turkey (McAfee et al., 2010). Processed meat is defined as any meat preserved by smoking, curing or salting, or with the addition of chemical preservatives. Examples are cured and smoked meats, ham, bacon, sausages, hamburgers, salami and tinned meat (Bouvard et al., 2015; Santarelli et al., 2008). Meat is the main source of proteins in our omnivorous Western diet, and red meat is known to contain essential nutrients such as iron, zinc, niacin, riboflavin, pantothenic acid, selenium, ω3-fatty acids, amino acids and vitamin B12 and B6 (McAfee et al., 2010; Oostinder et al., 2014). It contains antioxidants and other bioactive substances such as taurine, glutathione, creatine and choline (Williams, 2007). Consequently, the consumption of red meat can contribute to a complete, nutrient-rich diet. Nevertheless, a negative perception of red and processed meat is arising as a result of recent reports and messages in the media, stating that its consumption may increase the risk of certain diseases, next to posing a burden on agricultural systems and climate change. It is known that our diet is an important contributing factor with regard to the risk of some non-communicable diseases, which include cancer, cardiovascular diseases, obesity, diabetes and osteoporosis.

Especially colorectal cancer (CRC) is of concern, as more and more studies confirm that red and processed meat have an impact on the development of CRC. Different expert panels stated that there was substantial proof from cohort and case-control studies to accept a dose-response relationship between the consumption of red meat and cancer exists, and is supported by evidence for plausible mechanisms (Marmot et al., 2007). Support for this statement was found in large-scale epidemiological studies, such as the EPIC trial (European Prospective Investigation into Cancer and Nutrition), and the Continuous Uptake Project by the World Cancer Research Fund (WCRF). In a review of Aykan (2015) the results of 10 meta-analyses were evaluated, and it was concluded that red and processed meats significantly increase the risk of developing CRC by 20% and 30% respectively, whereas white meat does not have a significant effect. Also the IARC (International Agency for Research on Cancer) did a an extensive investigation of the carcinogenic effects of red and processed meat, in order to formulate a solid recommendation. They found conclusive evidence that there is a "positive association between eating red meat and developing CRC as well as strong mechanistic evidence". Therefore, the IARC classified **processed meat as a Group 1 carcinogen** ('carcinogenic to humans') and **red meat as a Group 2A carcinogen** ('probably carcinogenic to humans') (Bouvard et al., 2015).

There is a growing interest in unravelling the link between meat consumption and CRC, in order to provide solid, science-based guidelines for the consumption of red and processed meat, which could lead to an optimization of the intake of nutrients, and the reduction of the risk of CRC. The average red meat intake of a regular consumer is 50-100 g per person per day, with sometimes peak intakes of 200 g (Bouvard et al., 2015). In 2007 the WCRF formulated nutritional recommendations for the intake of meat, more specific (i) to switch from high fat to lean meat, (ii) to avoid processed meat and (iii) to limit red meat consumption to less than 500 g cooked red meat or processed meat products per week (WCRF, 2007). The association between the consumption of red and processed meat and the increased risk of CRC has been investigated in many ways in recent years, but remains uncertain. The relation is complex and depends on other factors than meat intake alone, such as the total composition of the diet, and genetic or environmental backgrounds (Oostinder et al., 2014).

Literature review

1 COLORECTAL CANCER AND LINK WITH MEAT CONSUMPTION

1.1 COLORECTAL CANCER

Colorectal cancer is an example of a non-communicable disease which is influenced by our eating habits and physical activity. It is of high interest because it is a major cause of morbidity and mortality throughout the world. CRC is the third most common cancer in the world, with nearly 1.4 million new cases diagnosed in 2012 (WCRF, 2015). It accounts for 9.4% of all cancers in men and 10.1% in women, and is the fourth most common cause of death (Haggar & Boushey, 2009). The mortality worldwide attributed to CRC is estimated half that of the incidence. Almost 55% of the cases occur in more developed regions. The highest incidence rates are found in Australia, New Zealand, the United States and Western Europe (with an average of more than 40 cases per 100.000 people), while the lowest rates are found in Western Africa and some parts of Asia (with less than 5 cases per 100.000 people) (Haggar & Boushey, 2009; IARC, 2015). A remarkable development is that the incidence is increasing rapidly in countries that have recently made the transition from a relatively low-income economy to a high-income economy, such as Japan, Singapore and Eastern European countries. The rates have at least doubled in these countries since the mid-1970s. This already suggests a link between the disease and lifestyle and explains why CRC is often called a 'disease of civilization' (Boyle & Langman, 2000; Janout & Kollárová, 2001). Important personal risk factors are age, inherited genetic risk, history of adenomatous polyps, history of inflammatory bowel disease and family history of CRC or adenomatous polyps. The importance of age is proven by a study of Edwards et al. (2010) that showed that more than 90% of the diagnoses of CRC between 1975 and 2006 occurred at the age of 50 years or older, and is translated into screening programs that focus on the age-group of 50-74 years old. Next, on the one hand, some environmental risk factors, such as nutritional practices, smoking, physical activity, obesity and heavy alcohol consumption contribute to CRC development (Gao et al., 2017; Haggar & Boushey, 2009). On the other hand, higher intakes of dietary fiber, green leafy vegetables, folate, and calcium have a protective effect against CRC (Society, 2015). This implies that a lot of the CRC incidences could be prevented by awareness of these lifestyle factors.

1.2 DEVELOPMENT OF CANCER

1.2.1 Structure of the colon and rectum

The large intestine consists of the colon, which is the first and major part, and the rectum, which is defined as the final six inches of the large intestine. They form a muscular tube with several layers. Closest to the lumen is the *mucosa* (the lining), which again consists of several layers (**Figure 1**). Next, there is a fibrous tissue layer called the *submucosa*. Around that layer, a thick layer of muscle is found, called the *muscularis propria*. On the outside, there are two layers of connective tissue: the *subserosa* and *serosa* (**Figure 1**).
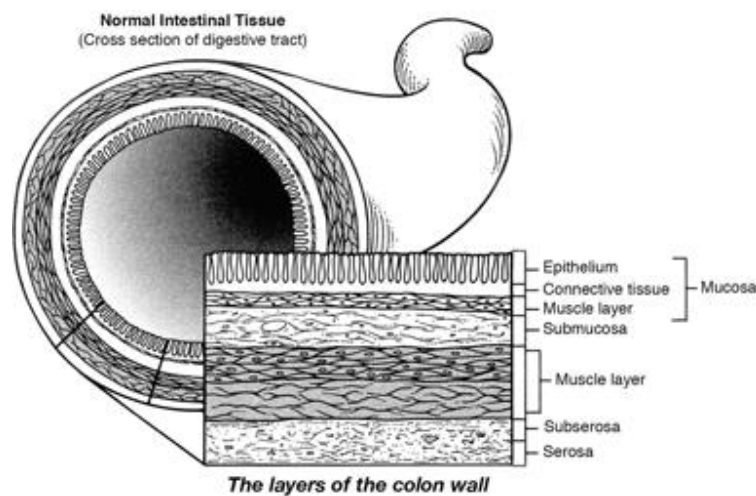

Figure 1: Structure of the colon and rectum wall ("Colorectal Cancer Staging," 2004)

1.2.2 Stages CRC development

Overall, CRC development is divided into five major stages ("Colorectal Cancer Staging," 2004; CTCA, 2017). **Stage 0** is the formation of Aberrant crypt foci (ACF) in the *mucosa*, which can further develop into pre-cancerous adenomatous polyps. **Stage 1** is the local invasion of the cells through the *muscularis mucosa* and into the *submucosa*, or even into the *muscularis propria*. **Stage 2** is the growth into and through the outermost layers of the colon or rectum, and even into nearby organs or tissues. In **stage 3** the cancer spreads even further and starts to affect lymph nodes. **Stage 4** is the most advanced stage of colorectal cancer. It means that the cancer has metastasized to distant sites, mostly the liver or the lungs. The formation of these secondary sites elsewhere in the body, is difficult to treat and the survival rates are rather low once this stage is reached.

The development of cancer is a multistep process, involving a series of histological, morphological, and genetic changes, occurring over several years. Most cancers (80-90%) arise from epithelial cells and are called 'carcinomas'. CRC typically starts off as benign polyps, arising from the inner lining of the colorectal wall. Only a small percentage of these polyps eventually becomes malignant. The underlying

cause of the transformation is a succession of genetic alterations within the cells, which result in a progressive evolution of normal cells into cancer cells, known as the adenoma-carcinoma sequence (Louis et al., 2014). The initiation of cancer is associated with specific mutations, e.g. in the APC (adenomatous polyposis coli), p53, K-RAS and B-RAF genes, resulting in some characteristic downstream consequences on cell growth, differentiation, motility and survival (Fearon & Vogelstein, 1990; Louis et al., 2014; Simon, 2016). Whereas genes that are responsible for the suppression of tumors and reparation of DNA are inactivated, proto-oncogenes are activated. More than a dozen core signaling pathways and processes responsible for tumorigenesis are affected (Parsons et al., 2008), all contributing to carcinogenesis. It is nowadays believed that there are three molecular pathways of genomic instability: the chromosomal instability pathway, the microsatellite instability pathway, and the hypermethylation pathway (Itzkowitz & Yio, 2004). Also intestinal inflammation and the gut microbiome may be involved in the development of CRC (Demeyer et al., 2016). The phenotypic effects are grouped into **six essential hallmarks**, and are used for characterizing cancer cells. These hallmarks are capabilities that the cancer cells acquire during the stages of cancer development. They include self-sufficiency in proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death (Hanahan & Weinberg, 2000).

1.3 CHANGING METABOLISM OF CANCER CELLS

There are fundamental differences in the core cellular metabolic pathways between normal cells and cancer cells. Many research is focused on understanding these changes, because this could improve tumor detection, cancer mitigation... Dividing cells have three basic needs: rapid ATP generation, increased synthesis of macromolecules, and maintenance of a stable redox status (Cairns et al., 2011).

To fulfill these needs in the stressful and heterogeneous environment of the tumor, adaptations to the metabolic pathways are necessary. Pushed by genetic mutations and the micro-environment, cells select alternative pathways that can generate enough ATP and other metabolites, so that the tumor cells are able to survive and even thrive in conditions that would be toxic for most normal cells (Amoêdo et al., 2013).

A frequently occurring adaptation is the so-called **Warburg effect**, which describes the shift from ATP generation through oxidative phosphorylation to ATP generation through anaerobic glycolysis, even when the oxygen supply is normal. The glycolysis provides ATP more rapidly, but is less efficient with regard to the ATP production per unit of glucose. As a consequence, the demand for glucose is much higher for tumor cells. Because this pathway is seen as a typical feature for cancer cells, it can be used to detect tumors or can form a platform for new therapeutic approaches (Amoêdo et al., 2013; Cairns et

al., 2011). The discovery of the Warburg effect initially led to the idea that mitochondria of tumor cells are dysfunctional organelles. Recently, this has been contradicted by data showing that the mitochondria are viable and respiring, and active participants in specific stages of the malignant transformation: a study of Chen (2012) has illustrated how mitochondrial oxidative stress could actively promote tumor progression and increase the metastatic potential of cancer cells.

Another observed effect is the **glutamine-addiction** of tumor cells (Amoêdo et al., 2013). Cells change to this higher consumption rate of glutamine as a consequence of specific mutations (for example in the RAS and MYC oncogenes, and in the p53 tumor suppressor gene) (Amoêdo et al., 2013; DeBerardinis et al., 2007). The cancer cells heavily depend on glutamine breakdown for the acquisition of building blocks for amino acids and other intermediates, which is extremely important given the rapid proliferation of the cells, that requires a constant supply of precursors of macromolecules. Moreover, glutaminolysis takes place, which is the entry of glutamine into citric acid cycle in the form of α-ketoglutarate (Ganapathy et al., 2009). Thereby it serves as a source of ATP and of NADPH, which then contributes to anabolic processes such as the synthesis of fatty acids and cholesterol (Amoêdo et al., 2013; Ganapathy et al., 2009).

Also in later stages of cancer development, metabolic changes take place. The early stages of CRC and other epithelial cancers are characterized by excessive cell proliferation and angiogenesis (Hanahan & Weinberg, 2000). In later stages of the multistep process of CRC, the cells acquire the final hallmark of cancer, namely invasiveness. Initially, they can invade through the basal membrane, which leads to metastasis, with life-threatening consequences. The exact mechanisms that lead to the acquisition of this invasiveness remain an area of intensive research. In many studies, an **epithelial-mesenchymal transition (EMT)** has been proposed as the key mechanism for the malignancy of epithelial cancer cells (Kalluri & Weinberg, 2009). The EMT is a biological process that allows polarized epithelial cells to undergo biochemical changes resulting in a more mesenchymal cell phenotype, which includes reduced cell-cell adhesion and therefore enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and increased production of extracellular matrix components. A key molecule in this process is the adhesion molecule (Epithelial) E-cadherin, as targeted disruption of this compound during tumor progression resulting in an inactivation of the cell adhesion system (Hirohashi, 1998), thereby facilitating the EMT.

The biochemical changes that go with EMT, also cause the cancer cells to de-differentiate and to acquire the characteristics of stem cells. They are then called **cancer stem cells (CSCs)**. Approximately 0.1-10% of all tumor cells are CSCs (Deonarain et al., 2009). It has been hypothesized that transformed stem cells can progress into intestinal adenomas and even seed secondary tumors (Garza-Treviño et al., 2015).

Compared to normal stem cells, CSCs are thought to show no restraint with respect to cell number (i.e. proliferation); however, their slow rate of cycling plays a role in resistance to treatment (chemotherapy and radiotherapy) and tumor recurrence (Moore & Lyle, 2011; Pannuti et al., 2010). CSCs are also characterized by a high adaptability, as more and more evidence suggests that they are a dynamic population of cells that are continuously shaped by a convergence of genetic, epigenetic, and micro-environmental factors (Kreso & Dick, 2014). Stemness thus seems to be a property that is highly dependent on the conditions wherein the cells live, next to being a cell-intrinsic property. All the influencing factors ultimately cause the diversity that is seen within tumor cells (inter- and intra-tumor differences).

The reason why the EMT takes place remains unclear. It is however certain that it is not a cell-autonomous process, which means that EMT requires signals from outside the cell to drive the transition (Bates & Mercurio, 2005). Recently, studies have shown that the micro-environment of the cancer cells is of importance, as growth factors such as epidermal growth factor (EGF), insulin-like growth factor-1 receptor (IGF-IR), fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF) or cytokines (TGF-β, TNF-α, IL-6) among others produced by the micro-environment can induce the de-differentiation (Garza-Treviño et al., 2015). The loss of adhesiveness, de-differentiation, and increased mobility and invasion are all hallmarks of increased malignancy, implying that the EMT provides a mechanism for cancer cells to acquire a more aggressive phenotype (Bates & Mercurio, 2005).

Another pathway that is activated in most tumors as a consequence of the different mutations linked to CRC is the **Wnt pathway**, which is therefore also a hallmark of CRC (J. Christensen et al., 2012). The Wnt signaling pathway is linked with the tumor suppressor APC and the protein β-catenin. In normal cells, free cytoplasmic β-catenin is destabilized by a complex containing APC, causing the β-catenin to degrade in further conversions (Roos-Mattjus & Sistonen, 2004). Cancer cells on the other hand are often characterized by mutations in the APC gene. It is found that in most colorectal tumors, both APC alleles are inactivated (Nagase & Nakamura, 1993). As a consequence, the APC complex is not formed in cancer cells, causing unusually high concentrations of β-catenin in the cytoplasm. The β-catenin is then translocated to the nucleus, where it acts as a co-activator in the transcription of Wnt target genes (Huelsken & Behrens, 2002; Molenaar et al., 1996), resulting in the activation of the Wnt signaling pathway. It is believed that this transcriptional response is critical in tumorigenesis (Korinek et al., 1997). The direct consequence is an increase in size of the proliferating crypt compartment, resulting in a polyp (Bienz & Clevers, 2000). The Wnt pathway is also influenced by the micro-environment, since Wnt proteins are secreted and are subsequently part of the tumor micro-environment (Clevers, 2006). Moreover, recant findings showed that high Wnt activity is an indicator of stemness (Vermeulen et al., 2010).

To summarize, it is nowadays thought that the key tumorigenic mutations occur in a few cells that possess the ability to self-renew and reside in tissues for a long time, eventually generating the hallmarks of CRC (Deonarain et al., 2009). The glycolytic energy generation, the EMT, Wnt activity and stemness are consequently considered as malignant traits (J. Christensen et al., 2012). Through these changes, tumor cell form highly adaptable units, allowing them to overcome the challenges that they face in the unfavorable environment, and even leading to resistance to regulatory events such as apoptosis (Amoêdo et al., 2013).

1.4 CANCER AND MEAT CONSUMPTION

Many research groups have focused on unravelling the mechanisms behind the colon cancer inducing effect. Compounds contributing to this effect can be summarized as follows: (i) meat matrix related factors such as heme-iron concentration, fat content; (ii) meat additives such as nitrite; (iii) compounds formed during meat preparation such as polycyclic aromatic hydrocarbons (PAH), heterocyclic amines (HCA), malondialdehyde (MDA), 4-hydroxynonenal (4-HNE); (iv) metabolites formed during gastro-intestinal digestion, such as N-nitroso compounds (NOCs), MDA, 4-HNE and (v) microbial metabolites, produced as a result of meat consumption, but not necessarily derived from the meat itself (Demeyer et al., 2016; Oostinder et al., 2014; Rombouts et al., 2017). These compounds have an impact on different stages of the colon cancer initiation and progression, as demonstrated in **Figure 2**.
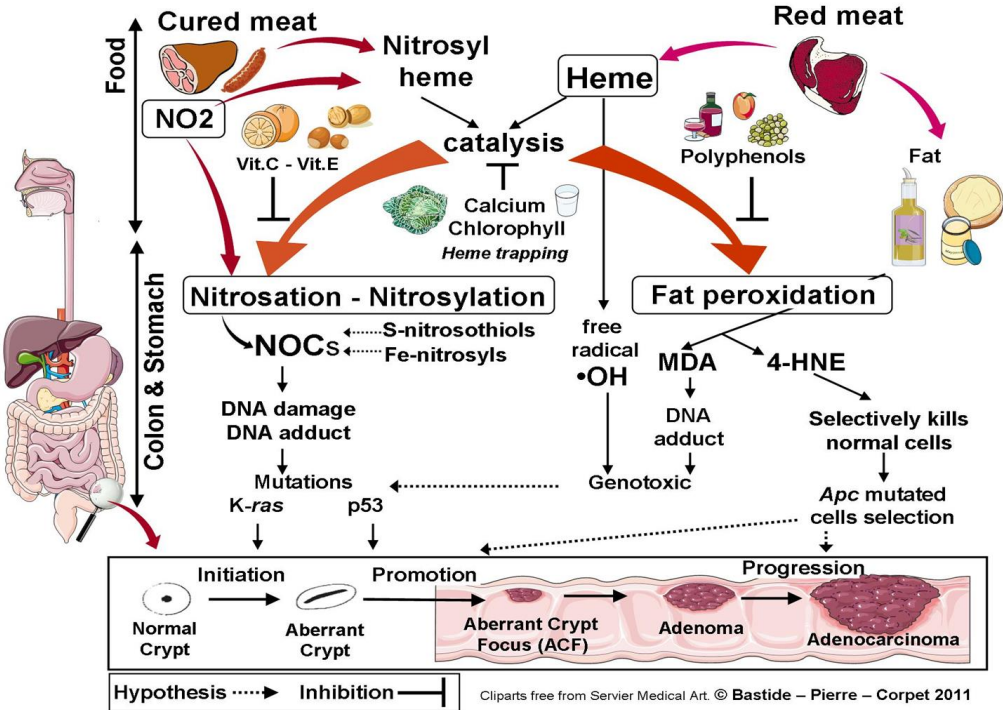

Figure 2: Mechanisms that connect red meat to the initiation and promotion of colorectal cancer (Bastide et al., 2011).

1.4.1   N-nitroso compounds (NOCs)

Meat is often cured to reduce spoilage during storage and increase food safety. Nitrate and nitrite are compounds that are used for this way of food preservation. Nitrite is an active curative agent as it limits growth of pathogenic microbes, controls oxidation and rancidity when added above certain thresholds and it causes a pink color that is appreciated by the consumer. Consequently, red meat is an exogenous source of NOCs, but it also contributes to the endogenous formation by providing precursors of NOCs and heme, that have a catalytic effect on NOC-formation in the stomach by a reaction between nitrogen oxides and secondary amines. Moreover, high temperatures during for example the preparation of meat can increase the NOC formation.

As mentioned above, tumor initiation is associated with mutations in for example the K-RAS and p53 genes. NOCs can play a role in these genetic alterations, as they yield alkylating agents that can react with the DNA of the target tissue. They are therefore considered as mutagenic agents, which can potentially initiate carcinogenesis (Saffhill et al., 1985). As a consequence of these findings, the residual nitrite content in cured meat products was decreased to approximately 10 mg/kg (10 ppm) for most products currently on the market. Also, the International Agency for Research in Cancer (IARC) classified "ingested nitrate or nitrite under conditions that result in endogenous nitrosation" as a group **2A carcinogen (probably carcinogenic to humans)**, in a wider context than meat intake alone (IARC, 2010). Still, the exact role of NOCs in the relation between red meat consumption and the increased risk of CRC is complex and that a lot of interactions take place.

1.4.2   Heme iron

Red meat is an important source of iron, as the bio-availability of the present iron is higher compared to other iron sources. The iron in red meat is embedded in the heme group, whereas in other food products, it is often present in its ionic form. In North America and Europe, two-thirds of the iron in the diet is present as inorganic iron and one-third as heme iron (Carpenter & Mahoney, 1992). However, two-thirds of body iron is derived from heme because much of the non-heme iron in the diet is largely bound to chelators, rendering it unavailable for absorption (Conrad & Umbreit, 2000). Heme consists of a porphyrin, which is a large heterocyclic organic ring, with in its center an iron atom in its ferrous form (Fe^{2+}). It is the prosthetic group of myoglobin and hemoglobin (Santarelli et al., 2008). Meat is our main source of heme, and red meat contains 10 times more heme than white meat (Schwartz & Ellefson, 1985). This argument is often used to demonstrate the role of heme, since an association is found for CRC and red meat consumption, but not for white meat. In red meat, heme is present in high concentrations in the form of myoglobin, resulting in the red color. There exist three types of heme: heme a, heme b and heme c. It is heme b that is part of the structure of hemoglobin and myoglobin

(**Figure 3**). In processed red meat, it occurs in its nitrosylated form because of the presence of nitrate and nitrite in the curing salt (Bastide et al., 2011).

Hemin is the oxidation product of heme b, and thus contains a ferric iron (Fe^{3+}) in the middle of the structure, which is additionally bound to a chloride ligand (**Figure 3**). It is a stabilized form of heme, that is used in research to simulate free heme in the diet.
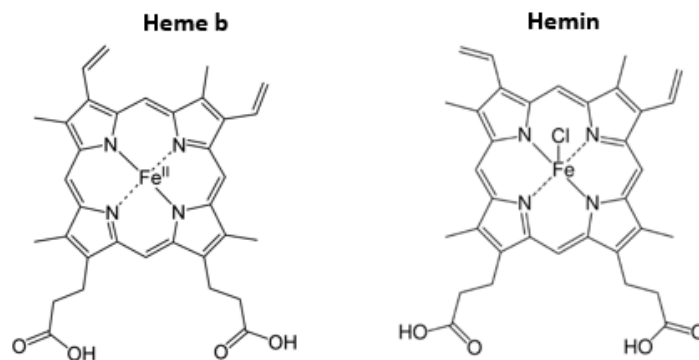

Figure 3: Chemical structure of heme b and hemin

Heme consumption has been linked to an increased risk of CRC by several epidemiological and experimental studies (Bastide et al., 2011; Lee et al., 2004; Qiao & Feng, 2013). After the action of enzymes on the meat, heme will be released into the gastrointestinal lumen. The luminal heme is taken up into the enterocytes as such, where the iron is released from the heme structure by heme oxygenase (Conrad & Umbreit, 2000). Nevertheless, the iron uptake in our body is highly regulated, so still a large portion of heme will pass the small intestine and reach the colon, especially if the iron balance is normal (Jeyakumar et al., 2016). Epidemiological studies report an increased risk of 14% (Qiao & Feng, 2013) and 18% (Bastide et al., 2011) for the group with the highest intake versus the lowest intake of heme. Experimental animal studies with chemically induced colon cancer have shown that dietary hemoglobin and red meat increase the number of aberrant crypt foci, putative cancer lesions and mucin-depleted foci (Fabrice Pierre et al., 2004).

Three mechanistic hypotheses have been put forward underlying the promotion of colorectal cancer by heme: (1) the catalytic effect of heme iron on the endogenous formation of NOCs, (2) the catalytic effect of heme iron on the formation of lipid oxidation products and (3) heme promoting colonic cytotoxicity in normal cells, but not in premalignant cells (Bastide et al., 2011; Sesink et al., 1999). However, the link between red and processed meat cannot be entirely ascribed to heme iron. One argument is that the content of heme in pork and chicken meat is similar (Lombardi-Boccia et al., 2002), while chicken consumption has no carcinogenic effect, in contrast to beef and pork (Bingham et al., 2002; Corpet, 2011; Santarelli et al., 2008).

1.4.3 Gut microbiota and the micro-environment

Mucosal cells are continuously in contact with the intestinal micro-environment which exists in the lumen of the colon. The large intestine normally contains over 10^{11} microbial cells per gram of content in healthy adults, and is dominated by anaerobic bacteria. A lot of inter-individual variability in microbial composition exists. Microbiota present in the lower intestinal tract form an important link between what we eat and the lumen conditions (David et al., 2014; Walker et al., 2011).

In previous studies, a relationship has been established between abnormal gut fermentation and diseases as obesity and cancer. The exact role or pathways are not clear, but there are several hypotheses that the microbiota could contribute to the relation between eating red meat and health. It was shown that patients with CRC have another composition of the gut microbiota, as well quantitatively as qualitatively (Demeyer et al., 2016). In a review of Louis et al. (2014), the conclusion was that the gut microbiota and particularly the cumulative effect of their metabolic products have a strong impact on the protection against, and predisposition to, CRC. Some microbial metabolites might suppress inflammation and cancer, such as short-chain fatty acids, while other metabolites might have the opposite effect and promote carcinogenesis. For example in patients with CRC less butyrate-producing bacteria were present, and potentially harmful bacteria increased in numbers, which may cause damage to the epithelial DNA, impair the gut barrier function or affect the host metabolism in a negative way (Oostinder et al., 2014).

A plethora of components is present in the lumen of the colon as a result of digestion and microbial fermentation. When eating red meat, these components will be different than when one eats white meat. It is the combination of food products that are digested that result in a certain environment. Some microbial metabolites that were identified are listed in **Table 1**. For example, a high intake of dietary fiber, was associated with a reduced risk of colorectal cancer. The hypothesis is here that the non-digestible carbohydrates increase the stool bulk and dilute the carcinogens in the colonic lumen, reduce the transit time, and stimulate the bacterial fermentation of fiber to short chain fatty acids (SCFA) (Lipkin et al., 1999). One SCFA that is of great interest is butyrate, as will be discussed further on. Other microbial products, such as kynurenine, have a negative impact on the micro-environment in the lumen, which may stimulate cancer development in its different stages.

Table 1: Microbial compounds involved in colon cancer development, based on Borges-Canha et al. (2015); Louis et al. (2014); Rombouts et al. (2017).

Dietary compounds	Microbial products	Effects
Non-digestible carbohydrates	Short chain fatty acids	Microbial modulation, cell differentiation, apoptosis, anti-inflammatory
Phytochemicals	Phenolic acids, isothiocyanates	Xenobiotic detoxification, microbial modulation, cell differentiation, apoptosis, anti-inflammatory
Proteins	NOCs, ammonia	Intracellular ROS production, genotoxicity
	Polyamines	Inflammation, intracellular ROS production, genotoxicity
	Hydrogen sulfide	Inflammation, intracellular ROS production, genotoxicity
Fat → Bile acids	Taurine	Microbial modulation
	Secondary bile acids	Microbial modulation, cell differentiation, apoptosis, ROS production, genotoxicity
Xenobiotics	Carcinogens	Intracellular ROS production, genotoxicity
Ethanol	Acetaldehyde	Intracellular ROS production, genotoxicity
Microbial toxins (e.g. *E.coli*)	Cyclomodulin	Genotoxic , cell-cycle progression, proliferation, differentiation, apoptosis
Tryptophan	Kynurenine	Tumor immune escape, proliferation, metastasis

1.4.4   Mitigation through diet

Our diet is a very powerful 'tool' to regulate our health. Some experimental animal and human studies indicate that mitigation of the stimulating effect of meat and meat products on the development of CRC is possible by increasing the amount of certain nutrients in our diet. A balanced diet including vegetables and fruits may contain enough antioxidants, such as alpha-tocopherol, to trap the ROS before they can cause damage. Also calcium could suppress the stimulating effect of beef on carcinogenesis (Fabrice H. F. Pierre et al., 2013), probably because of its chelating action on heme iron. Moreover, a higher intake of garlic and fibers is reported to relate to a lower relative risk for CRC (WCRF/AICR, 2016). Fibers have protective effects, as they increase the feces volume and can be fermented in the colon thereby changing the gut environment in a positive way.

2 CELL CULTURE AS IN VITRO MODEL FOR CANCER DEVELOPMENT

2.1 POSSIBLE WAYS OF STUDYING CRC

Human observational studies have to deal with a lot of confounding factors, are costly and difficult because of the long timescale of cancer development, and are subsequently unlikely to establish a causal link between meat consumption and CRC. Animal model studies can be used as an alternative. Rodents are the most-used models for tumor biology research (Oostinder et al., 2014). The animals can be made more susceptible to the development of tumors, which speeds up the process. A lot of research is done with nude mice, which have a genetic mutation causing them to lack a thymus, resulting in the absence of T-lymphocytes and thus a limited immune system (McIntyre et al., 2015). Other relevant models are pigs, because they have a very similar digestive system to humans, or primates, but those are expensive and have to deal with more complex ethical questions (Oostinder et al., 2014). Moreover, human colorectal cancer is known to be very diverse, which makes it impossible for one animal model to adequately represent all the forms (Johnson & Fleet, 2013). The end-point of these studies can be mortality, tumor incidence or earlier stages of tumorigenesis. Still, some issues remain with animal models, as it is difficult to follow the progression of the tumor and the experiments are rather intensive and not suitable for a screening of a large number of compounds. *In vitro* models offer a simplified method to investigate the underlying molecular mechanisms of tumor biology and the effect of meat (components) on a cellular level. The principle is to bring colon epithelium cells of cancer origin in culture, and study the effect of certain treatments. These *in vitro* models may support *in vivo* models in determining the effect of meat consumption (Bastide et al., 2015; Oostinder et al., 2014).

Cell models are subject to criticism, such as that they reflect the real *in vivo* tumor situation poorly as they lack interactions, and that they are not able to represent the heterogeneity of a real tumor (Bates & Mercurio, 2005). Nevertheless, they have proven their relevance, as cellular models have assisted in unraveling specific mechanisms and key players in a variety of pathways. For example, most of the current knowledge of the mechanisms and pathways involved in the EMT has been obtained with the use of *in vitro* cell models, by incubating them with a certain treatment for a short period of time (up to 72h), thereby demonstrating the role of specific proteins (Bates & Mercurio, 2005; Bhowmick et al., 2001; Ellenrieder et al., 2001). Our research group has successfully exposed intestinal cells in Transwell systems to SCFA during 14 days (Van Rymenant et al., 2017), but besides this study, the effects of long-term exposure are largely unexplored.

2.2 CELL LINES

Three cell lines were used in our experiments, representing different stages of colon cancer development: HT-29, Caco-2 and HCT 116, and differing in mutations in these genes, as will be discussed later on. Consequently, each cell line has its own characteristics regarding morphology, expression of differentiation markers, migration and potential to form metastases (J. Christensen et al., 2012).

2.2.1 Caco-2

The Caco-2 cell line is of human origin and its characteristics are nowadays well-known and well-described. They are epithelial cells from the colon tissue of a person with CRC in the stage of colorectal adenocarcinoma (ATCC, 2016a). The cells are tumorigenic, and express some keratin, retinol binding protein II and retinoic acid binding protein I. Caco-2 cells are adherent and form a monolayer (Levin & Davis, 1997).

The most important reason to make use of the Caco-2 cell line in the context of *in vitro* research is that these cells spontaneously show enterocytic differentiation in a long-term culture, which is ideal for adsorption and barrier studies. It is seen that approximately 14 days after reaching confluency, the cells express morphological and biochemical characteristics of small intestinal enterocytes (J. Christensen et al., 2012). The best way to reproduce the *in vivo* situation of the intestinal tract is using Transwell bicompartimental chambers. Nevertheless, it is also possible to use the Caco-2 cells in their undifferentiated form and as a monolayer in normal flasks. Undifferentiated Caco-2 cells show the strongest Wnt signal of the three cell lines discussed here (J. Christensen et al., 2012).

2.2.2 HT-29

The human colon adenocarcinoma cell line HT-29 offers a favorable experimental system for the study of factors involved in differentiation of epithelial cells. The HT-29 cell line is relatively similar to the Caco-2 cell line as the cells also originate from human colon epithelial tissue in the stage of colorectal adenocarcinoma. The cells are also adherent and were isolated from a primary tumor, causing them to have tumorigenic characteristics. In fact, differentiated HT-29 cells show the most similar gene expression patterns to tumor bulk derived cells (J. Christensen et al., 2012).

A variety of genes, antigens and receptors are expressed. Some interesting cellular products are formed: the secretory component of IgA, the carcinoembryonic antigen (CEA), the transforming growth factor beta binding protein and mucin. Mainly, they produce metabolites, cytokines, growth factors, etc., which are known to promote cell survival (Martínez-Maqueda et al., 2015).

The line is positive for expression of C-MYC, K-RAS, H-RAS, N-RAS, MYB, SIS and FOS oncogenes, and the p53 antigen is overproduced (ATCC, 2016c).

Under standard conditions, the cells grow as a non-polarized, undifferentiated multilayer. However, when the culture conditions change (e.g. sugar/glucose-free medium), a differentiated and polarized morphology can arise, which increases the similarity with intestinal epithelial cells (Zweibaum et al., 1985). The time course of the differentiation is longer compared to Caco-2 cells: 30 days vs. 15-20 days for the Caco-2 cells. Also, the levels of enzymatic activities are lower than in Caco-2; lactase is absent; and only 40–50 % of HT-29 cells express sucrose-isomaltase. However, one of the main differences between this cell line and Caco-2 is that HT-29 cells can produce mucin at a relatively high level when they are transformed into mucus-secreting differentiated cells, e.g. by low shear stress (Cohen et al., 1999; Martínez-Maqueda et al., 2015).

2.2.3   HCT 116

The HCT 116 cell line is also from human colon tissue origin. These cells are isolated from an adult male with CRC in the stage of colorectal carcinoma. They are adherent and highly tumorigenic. The cells produce carcinoembryonic antigen (CEA) and keratin, which can be detected by immunoperoxidase staining. This line has a mutation in codon 13 of the RAS proto-oncogene and expresses the transforming growth factor beta 1 and 2 (ATCC, 2016b). HCT 116 is a growth factor-independent cell line that has been shown to be invasive and highly motile in *in vitro* studies (Rajput et al., 2008). This high invasiveness is a consequence of the EMT, that is strongly present in this cell line (J. Christensen et al., 2012).

Objectives

The starting point of this master thesis is the hypothesis that red/processed meat consumption and hence digestion, modify the micro-environment in the colonic lumen and lead towards the generation of a variety of metabolites, that, besides their effect on DNA-damage, may contribute to a more carcinogenic phenotype of intestinal cells and eventually to CRC development on the long term. As previously discussed, cancer cells develop different strategies to survive in the stressful environment of the tumor by modifying some core pathways. These adaptations help the cells to thrive and allow the cancer to evaluate through the different stages of colon cancer development. Changes in the metabolism are characteristic for malignancy, and can therefore be used for cancer detection, but also cancer mitigation strategies could focus on these pathways. Hence the necessity of understanding these pathways. The presence of certain molecules could contribute to these adaptations. With regard to red and processed meat consumption, these metabolites may be nitro(sy)lated compounds, lipid oxidation products, microbial products or other currently unidentified metabolites. Components associated with the consumption and digestion of red/processed meat (and not white meat) were tested on short term for their acute effect, and longer term to study their effect on processes included in later stages of CRC progression, which is largely unexplored. As human intervention studies with CRC development as an endpoint are not justifiable, *in vitro* cell line based techniques were used and a long-term approach was optimized. The effect on cell viability, cell numbers, cytotoxicity, mitochondrial respiration and oxidative stress were assessed. The objective is thus to validate a new long-term technique of investigating CRC development *in vitro*, and to explore the impact of specific components on some key cellular pathways.

Materials and methods

Five components were selected based on their link with the consumption/digestion of red meat and/or colorectal cancer: nitrite (a meat additive), hemoglobin, hemin (both meat matrix related factors), sodium butyrate (microbial fermentation product derived from dietary fibers), and kynurenine (microbial metabolite produced as a result of meat consumption) (Aykan, 2015; Demeyer et al., 2016; Oostinder et al., 2014; Rombouts et al., 2017). Two setups were tested. First, cells are exposed to a large range of concentrations of the chosen components for a short period of time. With the obtained information, a second phase was started up, where cells are exposed to sub-toxic concentrations of the three components that had a significant effect for a longer time.

1 BIOASSAYS

1.1 CELL CULTURE

1.1.1 Cell maintenance

Three commercially available cell lines from human origin were used: Caco-2, HT-29 and HCT 116. The cells were obtained from the American Type Culture Collection (ATCC) and were kept in culture in 25 cm² or 75 cm² Sarstedt Cell Culture Flasks (Nümbrecht, Germany). The bottom surface of the flasks is tissue-culture treated to assure an optimal attachment of the cells, and are equipped with a vent cap, so that gas exchange is always possible. The medium that was used, consisted of Dulbecco's modified Eagle's medium (DMEM) + Glutamax (Life Technologies, Ledeberg, Belgium), 10% fetal bovine serum (FBS) (Greiner bio-one, Wemmel, Belgium), 1% Penicillin/Streptomycin (Life Technologies) and 1% non-essential amino acids (NEAA) (Life Technologies).

1.1.2 Splitting

At a confluency of 70 to 80%, the cells were split in a ratio depending on the cell line and the conditions (ranging from 1/3 to 1/10). To detach the cells from the bottom, trypsin-EDTA (Life Technologies) was used, a proteolytic enzyme that is inhibited by Ca^{2+} and Mg^{2+} ions. Therefore, a washing step with PBS was always done first. In a low concentration and controlled time-temperature process, mostly around 5 minutes at 37 °C, trypsin cuts the proteins that are responsible for the cell-surface adhesion and cell-cell adhesion, and a single cell suspension can be obtained.

For the culture flasks (25 cm²), 4 mL of PBS and 2 mL of trypsin was added. In the long-term experiments, the cells were also trypsinized in 12-well plates upon confluency, but there, 1 mL PBS and 300 µL trypsin per well was used.

1.1.3   Counting cells

To ensure a reproducible seeding concentration, and to standardize data based on cell number, it was warranted that the number of cells in a suspension was known. Cells were counted in a Bürker counting chamber. The cell suspension was mixed with Trypan Blue (0.5 g/100 mL, Sigma) in a 1:1 ratio. Only dead cells are permeable for Trypan blue and take up the dye, so that they can be distinguished from the living cells. Cells were counted visually using a Motic phase-contrast microscope (VWR, Leuven, Belgium).

1.2   MTT ASSAY

The MTT assay is based on conversion of water soluble, yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) compound to the insoluble, blue formazan. Viable cells with **mitochondrial activity** can do this conversion, however, dead cells lose this ability. The measured absorbance (590 nm) is thus proportional to the number of viable cells. Cells were seeded on 96-well plates with a medium volume of 200 µL and a minimum seeding concentration of 20 000 cells per well. At 2 or 3 days after treatment, the assay was performed. 100 µL cell culture medium was removed from the wells, and 20 µL MTT (5 mg/mL MTT in PBSD-, filtersterilized with a 20 mL syringe through a 0.22 µm filter) was added to each well. After incubating in the dark during 2 hours at 37°C, the liquid was removed. Blue formazan crystals stick to the bottom. They were suspended in 200 µL DMSO (dimethylsulfoxide, Sigma-Aldrich, and the absorbance was measured at 570 nm with a multireader (Spectramax, Molecular Devices, Berkshire, UK). Six wells that did not contain any cells, were filled with 200 µL DMSO to serve as blanc.

1.3   SRB ASSAY

The SRB assay relies on the property of Sulforhodamine B (SRB), which binds to amino acid residues of proteins in the cells. The amount of bound SRB is then a proxy for **cell number** which can subsequently be extrapolated to measure cell proliferation. Again, the assay was performed on cells that were seeded on 96-well plates as described in the MTT assay, and incubated for 2 or 3 days. The cells were then fixated by the addition of 50 µL trichloroacetic acid (50%TCA in MilliQ®-water), after which the plate was placed at 4°C for 1 hour. Subsequently, the plate was washed 5 times with tap water and dried to the air. Next, 50 µL SRB solution (0.4% in 1% glacial acetic acid) was added to stain the cells. After 30 minutes, the plate was rinsed with 1% glacial acetic acid in MilliQ®-water and dried.

Next, 200 μL of a 10 mM Tris-buffer (tris(hydroxymethyl)aminomethane) was added and the liquid was suspended until a homogenous solution was obtained in each well. Finally, the absorbance was determined at 490 nm with a multireader. Some empty wells filled with 200 μL of Tris-buffer served as blanc.

The MTT and SRB assays were always combined, as the two tests together provides the most useful and interpretable information, as is shown in **Table 2**.

Table 2: Interpretation of the MTT and SRB assay

MTT	SRB	Most probable interpretation
↑	=	Cells show reactivity (or stress) without toxicity
↓	= or ↓	Toxicity
=	↓	Secretion of proteins or cell release
↑	↑	Cell proliferation
=	↑	Adhesion of protein-like compounds on the cells

1.4 LDH ASSAY

The Pierce LDH Cytotoxicity Assay Kit measures the amount of lactate dehydrogenase (LDH), which is released into the cell culture medium when the plasma membrane of the cells is damaged. The absorbance is directly proportional to the amount of LDH released, which is indicative of **cytotoxicity**. The reagents are prepared starting from the kit content: Substrate Mix, Assay Buffer, 10X Lysis Buffer, Stop solution, LDH Positive Control. A Reaction Mixture is prepared by dissolving the Substrate mix with 11.4 mL MilliQ®-water, and combining this solution with 0.6 mL of Assay buffer. The resulting mixture should be protected from light.

Cells are seeded on 96-well plates and treated with the chosen conditions. Then, the assay was performed by first transferring 50 μL of the medium of each well to a new 96-well flat bottom plate. Next, 50 μL of the Reaction Mixture was added. After 30 minutes incubation at room temperature and protected from light, 50 μL of Stop solution was pipetted into each well. Finally, the absorbance was measured at 490 nm and 680 nm. To determine the LDH activity, the absorbance value at 680 nm should be subtracted from the 490 nm absorbance value.

For LDH tests on plates treated with hemin, the dilutions of hemin in cell culture medium were used as blanc. This was necessary as hemin has a dark brown color, which influences the color of the medium, and thus the absorbance, especially for the higher concentrations.

1.5 GLUTATHIONE ASSAY

The glutathione assay gives an idea of the overall **redox status** of the cells after a treatment. Therefore, this assay quantifies the reduced (GSH) and oxidized (GSSG) form of glutathione, which is the most abundant redox couple in a cell (Schafer & Buettner, 2001). The ratio GSSG/GSH then gives an idea of the oxidative stress within the cells (which are lysed first to release all cell content). Cells that were seeded on 96-well plates were washed with PBS and homogenized with 200 µL of 20 mM Tris solution with 0.1% Triton X100. Three new black 96-well plates with clear bottom (Greiner) were prepared, one for the GSH quantification, one for the GSSG quantification, and one for a Bradford assay.

For the GSH quantification, the plate was kept on ice during the assay to avoid oxidation. 10 µL of the cell homogenate was transferred to the new plate and 10 µL buffered formaldehyde (37-40% formaldehyde/ 0.1 M phosphate buffer, pH 8 in a 1/4 ratio) was added. After incubating at room temperature for 5 minutes, 170 µL phosphate-EDTA buffer (phosphate buffer 0.1 M – 5 mM EDTA, pH 8) and 10 µL OPT (o-phtalaldehyde, 1 mg/mL in absolute methanol, prepared daily and protected from light) were added. After 45 minutes, the excitation at 355 nm and emission at 460 nm was recorded with a fluorimeter (Spectramax).

For the GSSG quantification 10 µL of the cell homogenate was transferred to a new black well plate and 10 µL buffered formaldehyde was added. After 5 minutes, 4 µL of NEM was added and this was incubated for 30 minutes at room temperature. Next, 176 µL of 0.1 NaOH and 10 µL OPT were added and the mixture was incubated for 45 minutes at room temperature. Finally the fluorescence at an excitation wavelength of 355 nm and emission wavelength of 460 nm was determined.

1.6 BRADFORD

The Bradford assay is a spectroscopic procedure used to measure the **protein concentration** in a solution, and in an indirect way, to estimate the number of cells in a solution. The Bradford reagent consists of Brilliant Blue G dye in phosphoric acid and methanol. The protein-dye complex causes a shift in the absorption maximum of the dye to 595 nm.

To free the proteins from the cells, a lysis step was performed. Cells that were seeded on 12-well plates, were trypsinized and suspended in 1 mL of cell culture medium. An aliquot hereof was transferred to an Eppendorf tube. The Eppendorf tubes were then centrifuged for 5 minutes at 4000 rpm using a Eppendorf centrifuge (Spectrafuge, Sigma). The supernatants was removed and the pellet was lysed with 100 µL of 0.1% Triton X100 buffer (to determine the optimal volume of lysis buffer, an optimization

experiment was first performed as discussed in the results). These lysates were then suitable to perform the Bradford assay.

The standard protocol included the transfer 5 μL of the unknown protein samples, or lysis buffer as blanc, to a 96-well plate in six-fold and the addition of 250 μL Bradford reagent. After 30 minutes, the absorbance was measured at 595 nm.

In order to have a quantitative measure for the protein concentration using the Bradford assay, a BSA standard curve was established. A series of known concentrations of BSA (Sigma) protein between 0 mg/mL and 1.4 mg/mL was made and the Bradford quantification was performed on each of these dilutions. This resulted in a curve showing the absorbance in function of the protein concentration (mg/mL). Next, a dilution series was made of every cell line ranging in Eppendorf tubes, with each tube containing a defined number of cells, ranging from 0 to 2 million cells. The lysis and Bradford protocol was performed, resulting in a calibration curve for each cell line showing the absorbance in function of the number of cells. By combining the two curves, a calibration curve showing the protein concentration (mg/mL) in function of the cell count was created, one for every cell line, that was used during all the experiments.

1.7 SEAHORSE

The XF24 Analyzer (Seahorse) is a probe-based system able to measure parameters involved in the aerobic mitochondrial respiration (**O₂ consumption**), or anaerobic energy production after glycolysis in the cytosol (**pH decrease**), on an on-line basis. The system is provided with an injection module, that allows to automatically inject four treatments during the course of the Seahorse-run. This way, basal respiration, as well as respiration changes after a set of inhibitors/stressors, can be investigated. The company has developed several stress kits, that allow to study the mechanism by which respiration and glycolytic processes are disturbed. The assay that was used in this project, was the MitoStress kit. This kit contains oligomycin as an inhibitor of ATP synthase and thereby disrupting oxidative phosporylation, and is therefore a measurement of ATP production. Next, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) is added to the cells, which is an uncoupling agent that collapses the proton gradient, disrupts the mitochondrial membrane potential, thereby facilitating electron transport, and thus maximal respiration. Finally, antimycin/rotenone is added, which shuts down mitochondrial respiration by inhibiting complex I and III, and the rest oxygen consumption is then the result of non-mitochondrial respiration. A typical response of cells towards these compounds is depicted in **Figure 4**.

Seahorse XF Cell Mito Stress Test Profile
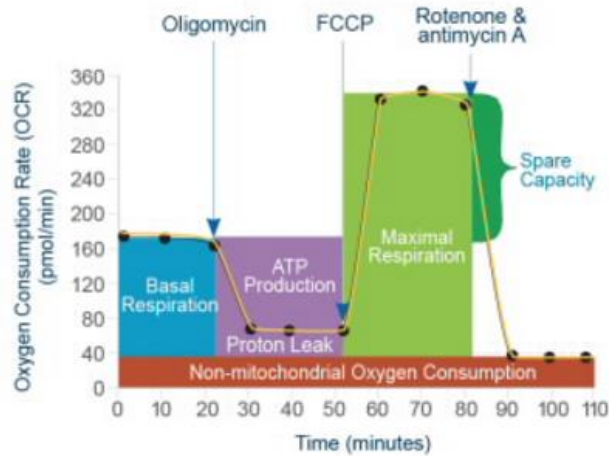### Mitochondrial Respiration

Figure 4: Overview of the biological implication of the addition of the compounds of the MitoStress kit (Agilent, 2017).

In this project, we wanted to investigate whether a long-term pretreatment with butyrate, hemin and kynurenine, had an impact on the Warburg effect, which is a shift from aerobic mitochondrial respiration, towards anaerobic lactate production in a more cancer-like cell phenotype. Therefore, we have harvested and counted HT-29 cells from the second long-term experiment, in which the cells were pretreated with butyrate, hemin and kynurenine for 12 days in four concentrations. A seeding density of 30.000 cells per well was used. Next, the cells were grown in special 24-well plates for one day in their normal growth medium supplemented with the treatment, at 10% CO_2 and 37°C conditions. In the meantime, the XF24 probe-containing cartridge was hydrated overnight in XF calibrant solution at 37°C under normal air conditions, and in a plastic bag with wet tissue to avoid evaporation of the calibrant solution.

The next day, non-buffered and glucose-free XF-base medium was supplemented with 2 mM glutamine and 1 mM pyruvate and the pH was adjusted to 7.4 in a water bath at 37°C. This medium was used to wash the cells (twice, 1 mL each time), and then the medium in each well was adjusted to approximately 450 μL. The cells were then incubated at 37°C under normal air (in a plastic bag with wet tissue) according to the manufacturer's instructions, for one hour prior to the assay. In the meantime, stock solutions of glucose, and compounds of the MitoStress Kit (Seahorse), including oligomycin, FCCP and antimycin/rotenone were prepared and transferred to the four injection ports of the cartridge per well. Stock solutions and injection volumes were prepared so that the concentrations after sequential addition in the wells was 25 mM for glucose (A), 1 μM for oligomycin (B), 1 μM for FCCP (C) and 0.5 μM of antimycin/rotenone (D). The assay run was for approximately 120 minutes per plate, and consisted of a calibration step of the probes, monitoring of the basal respiration, injection of glucose, oligomycin, FCCP and antimycin/rotenone. For the basal respiration, the oxygen consumption (OCR) and extracellular

acidification (ECAR) were measured 3 times during 2 minutes, with 3 minutes interval between the measurements, and this was the same after each injection of the assay compound A to D. **Figure 5** gives a representative OCR profile with indication of the injection times and compounds. In one day, 4 plates were run (two plates of replicate 1 in the morning, two plates of replicate 2 in the afternoon, approximately 5 hours between first and second replicate), so that each condition could be duplicated (= technical replicate, but with time difference of 5 hours). Per technical replicate, 3 biological replicates were randomly seeded. A biological replicate was defined as one sample from an individual batch of cells that had been treated with the compound of interest during 12 days, so the 3 biological replicates were not taken from the same batch of cells, but from 3 different batches so that the maximal variability, induced by long-term treatment, was retained. After analysis, an SRB test to determine the protein content was applied to normalize the response to cell density, and this value was also used as a parameter to exclude some measurements of which the cell density was too low to be measured adequately.
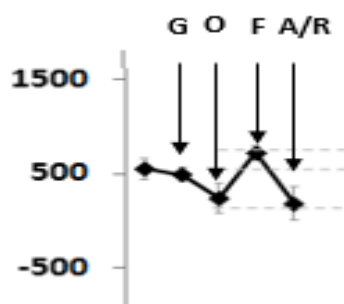

Figure 5: Characteristic profile of the oxygen consumption rate (OCR) a normal responding cell on glucose (G), oligomycin (O), FCCP (F) and antimycin/rotenone (A/R).

1.8 INCUCYTE® LIVE CELL ANALYSIS

IncuCyte is a live-cell analysis system that automatically acquires images of (theoretically) 600 wells by scanning each well every two hours, providing real-time kinetic data.

HT-29 cells were seeded on two 96-well plates (Costar®). To avoid the need to split the cells after a few days, the seeding density was kept very low (4000 cells per well). After the seeding, the plates were kept in the IncuCyte incubator at 37°C and 5% CO_2 for 48 hours with normal medium. After this settling time, the treatments were applied. 12 conditions were tested in sixfold, and the untreated cells were repeated 24 times. The outer columns and rows of the plates were filled with sterile PBS to avoid edge effects. Every two days, the medium was changed with fresh serum-containing medium, including the selected component. After 12 days, the experiment was ended and the images were analyzed with the IncuCyte® Software.

1.9 CLONOGENIC ASSAY

In parallel with the IncuCyte experiment, a clonogenic assay was performed on the HT-29 cells. This is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony, which is a measure for its ability for 'unlimited division'. HT-29 cells were seeded in 6-well plates (Thermo Scientific) in a density of 500 cells per well. After 48 hours of incubation at 37°C and 5% CO_2, the 13 conditions (12 treatments and 1 reference) were applied in duplicate. After 12 days, the medium was removed and the cells were washed 2 times with 2 mL PBS. The colonies were fixed and stained with a solution of formaldehyde and filtered crystal violet (12.5 crystal violet, 250 mL 40% formaldehyde, 750 mL 100% ethanol, 4.25g NaCl, aquaDest until 2.5L). The plates were put on a shaker for 15 minutes, after which each well was washed 5 times with water. Next, the plates were dried for 24 hours at room temperature, before the colonies were counted using a stereomicroscope (Nikon/SMZ1270, Brussels).

2 EXPERIMENTAL SETUPS

2.1 SHORT-TERM

For the short-term experiments, 20 000 cells per well were seeded on flat bottom 96-well plates (Greiner). The cells were left under normal circumstances (with normal, serum-containing medium) in the incubator (Memmert, VWR) at 37°C and a CO_2-concentration of 10% for 24 hours, so that they could attach to the bottom of the plate, before treating them with one of the chosen compounds. To avoid edge effects, only the inner 60 wells of each plate were used to seed the cells, whereas the wells at the edge were filled with PBS.

Unless stated otherwise, cells were incubated for 24 hours under serum-containing medium, and then treated with the test-compound in serum-free medium for three days. Six wells of each cell line with each 20 000 cells are treated with normal serum-free medium, to serve as the reference.

After three days of treatment, a series of endpoint assays are performed. These include an MTT assay, SRB assay, LDH assay and glutathione assay. A schematic overview of the set-up is represented in **Figure 6**.
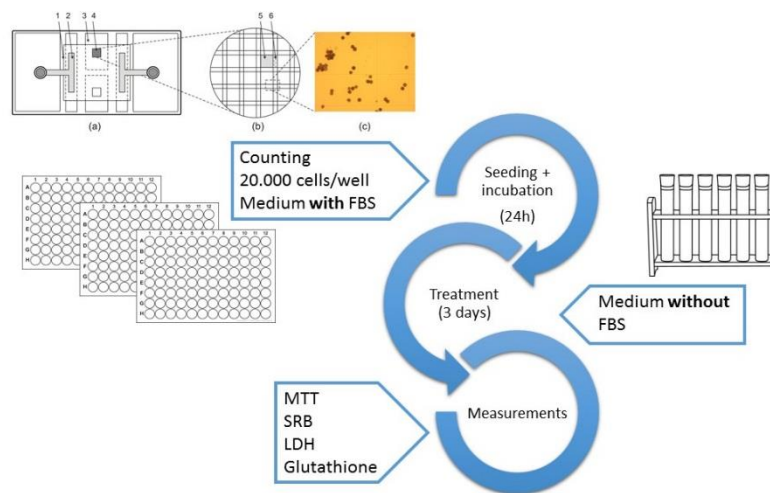
Figure 6: Overview of the short-term exposure set-up

2.2 LONG-TERM

In a second phase of the research, the same three cell lines were used to further investigate the effect of the components that gave the most interesting results for the short-term exposure. Two trials of long-term exposure were performed. Cells were seeded on 12-well plates or 6-well plates (Corning, Elscolab, Belgium). To seed the cells at the same 'cells per surface area' ratio as in the short-term tests, 237 500 cells (12-well plate) or 600 000 cells (6-well plate) per well are needed.

During the long-term approach, every two days, the medium was renewed by aspirating the old medium and adding fresh medium, containing the component that was tested in that well. When reaching 60-80% of confluency, the cells were trypsinized and split. When single cells were formed, 1 mL of the component-medium solution was added to suspend the cells. 200 μL hereof was transferred to a new 12-well plate, so the cells were 1/5 diluted again, and the experiment was continued. In parallel, an aliquot of 0.5 mL of each well was taken in an Eppendorf tube, to determine the number of cells in each well using the Bradford assay. This resulted in a measurement of the absorbance for each condition. Together with the calibration curves that were established, an estimation of the number of cells in each well was derived, taking into account the dilutions that have been made.

Some endpoint measurements were included at the end of the second long-term experiment. This was done by transferring 20 000 cells from each condition of the long-term experiment onto a 96-well plate in six-fold, and treating these cells for three more days with the component of choice, so that enough cells were present for the assays. After three days, an MTT assay, SRB assay, LDH assay and glutathione assay were performed.

The Seahorse assay was done with HT-29 cells that originate from the second long-term exposure experiment. Moreover, an IncuCyte experiment and a clonogenic assay with HT-29 cells were set up in de laboratory of Prof. De Wever (Laboratory of Experimental Cancer Research) to validate the results of the new long-term protocol.

3 STATISTICS

Data shown in the figures represent mean values ± the standard deviation. The data was exported to IBM SPSS Statistics 24, where it could be further analyzed. Per cell line and per assay that was performed, the results were ordered per condition, that was mostly tested in three- or six-fold. The dependent variable was thus the concentration of the component, whereas the independent variable was the absolute value of the results of the assay. The homogeneity of variances was checked with the Levene test, before performing One way ANOVA to compare the mean values of the assay results. A post hoc Tukey HSD test was done to investigate which values significantly differed on a 0.05 significance level. If the assumption of homogeneity of variances was not fulfilled, the post hoc test Tamhane's T2 was chosen to still get an idea of significant differences. This procedures was repeated for each component and each performed assay.

Results

1 SHORT TERM

The objective of the short-term toxicity tests was to investigate the behavior of cells when treated with a compound related to the consumption of red meat and colorectal cancer. The concentrations at which these components were toxic, induce differentiation or cell proliferation, or do not have any effect at all, were of interest to determine a sub-toxic concentration-range for the long-term exposure experiments.

1.1 TREATMENTS

A first compound was **sodium butyrate** (Sigma), a short chain fatty acid formed by microbial fermentation of undigested fibers in the colon. Several studies show that butyrate has anti-tumor characteristics as it prevents proliferation and invasion of the epithelial cells, promotes differentiation and induces apoptosis in the cancer cells (Barnard & Warwick, 1993; Yu et al., 2010; Jintao Zhang et al., 2016). Although this microbial metabolite is not necessarily linked with red/processed meat, it may be considered as a negative control to ensure proper interpretation of the developed long-term approach for cancer development.

A second series of treatments was done with **hemoglobin**, a protein containing four heme groups transporting oxygen through the body. As described before, heme has been linked to an increased risk of CRC in several studies, but the exact role remains unclarified. Also **hemin** (Sigma), a chlorinated heme group, was used as a treatment for the cells, because this approaches the structure remaining after intestinal digestion better.

A fourth compound that was tested was **nitrite**. Nitrite is used in meat products as a preserving agent and to ensure that the color of the meat stays optimal.

A last component that was tested, was **kynurenine** (Sigma). Kynurenine is one of the 22 metabolites that were identified using MS-based fingerprinting of *in vitro* colonic digests of red meat by Rombouts et al. (2017). It is an intermediate in the katabolic pathway of tryptophan (Munn & Mellor, 2007; Rombouts et al., 2017).

An overview of the different conditions tested on the short-term is given in **Table 3**. The components were dissolved in the culture medium and added to the cells as described in the normal culture protocol.

Table 3: Overview of the conditions applied in the short-term experiments

Component	Cell line	Concentration (µM)								
Butyrate (based on Jintao Zhang et al. (2016))	Caco-2	500	5000	10000						
	HT-29	500	5000	10000						
	HCT 116	500	2500	5000	7500	10000	12500			
Hemoglobin (based on Glei et al. (2006))	Caco-2	50	100	200						
	HT-29	50	100	200						
	HCT 116	6.25	12.5	2	50	100	200			
Nitrite (based on Jädert et al. (2014))	Caco-2	1	100	1000						
	HT-29	1	100	1000						
	HCT 116	0.1	1	10	100	1000	10000			
Hemin (based on Glei et al. (2006)) – first try-out	Caco-2	1	5	10	50					
	HCT 116	1	5	10	50					
Hemin (based on Glei et al. (2006))	Caco-2	0.1	0.25	0.5	1	2.5	5	10	25	50
	HT-29	0.1	0.25	0.5	1	2.5	5	10	25	50
	HCT 116	0.1	0.25	0.5	1	2.5	5	10	25	50
Kynurenine (based on Diegelmann et al. (2012))	Caco-2	0.25	0.5	1	2.5	5	10	25	50	100
	HT-29	0.25	0.5	1	2.5	5	10	25	50	100
	HCT 116	0.25	0.5	1	2.5	5	10	25	50	100

1.2 TREATMENTS WITH NITRITE AND HEMOGLOBIN

Cells treated with nitrite showed no or very little changes. No effect was seen on cell viability (MTT), cell number (SRB) or cell toxicity (LDH) for all three cell lines, even at very high concentrations up to 10 mM, which are unrealistic for the colonic lumen situation.

The same applies for cells that were treated with hemoglobin. The MTT assays proved that there was no effect on mitochondrial respiration. The slight increase of the SRB values of Caco-2 cells could point to adhesion of protein-like components, such as hemoglobin itself. For this reason, hemin is probably a better compound to test the effect of the heme group.

1.3 TREATMENT WITH BUTYRATE

For the experiments with butyrate, some deviations in timing from the standard short-term protocol (24 hours incubation with normal medium, 3 days incubation with the compound) were applied. For the experiment with HT-29 cells, the cells grew for 48 hours in normal conditions and 48 hours under influence of the compound. The HCT 116 cells were exposed to the compound after 24 hours but only for 48 hours.

The results of the MTT, SRB and LDH assays are shown in **Figure 7**. Butyrate treatment significantly decreased the mean absorbance in the MTT and SRB assays for all three cell lines, in a dose-dependent way. For HT-29 cells and Caco-2 cells, the amount of LDH in the medium did not increase more than 20%, therefore, butyrate did not damage the plasma membrane of these cells, and the drop in MTT and SRB values was not caused by cytotoxicity. In contrast, the LDH values of HCT 116 cells increased up to 250% of the untreated conditions at a butyrate concentration of 12.5 mM, and even the lowest concentration already caused a 50% increase. Although this could mean that butyrate is toxic for HCT 116 we rather think that HCT 116 cells, which have the highest metastatic behavior of the three cell lines, detached from the monolayer into the medium, thereby creating an extra pool of LDH when the assay is performed on the uncentrifuged medium.
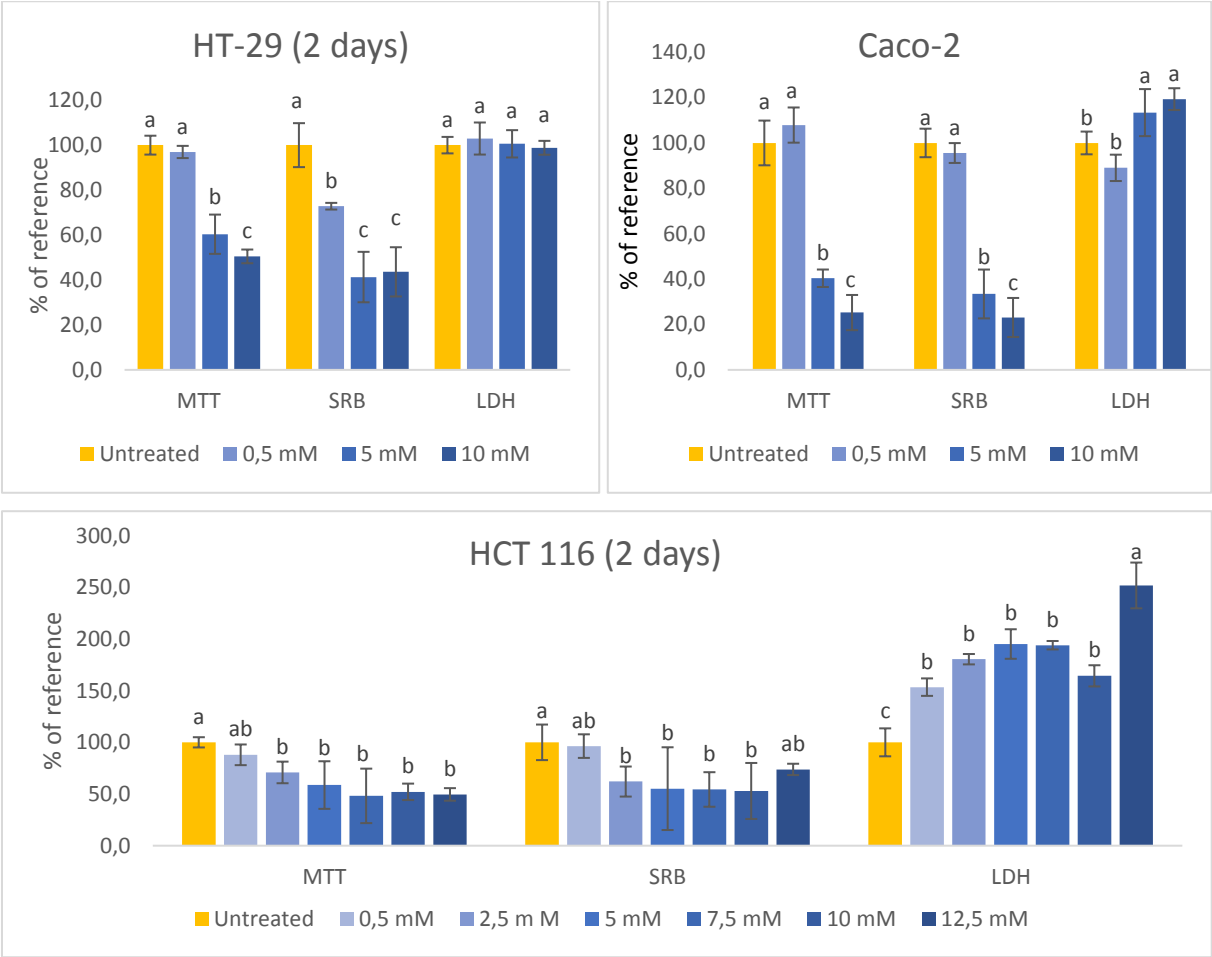

Figure 7: Results of the MTT, SRB and LDH assays for the cells treated with sodium butyrate. Mean values grouped with a different letter are significantly different from each other, based on a One Way ANOVA test (p<0.05).

1.4 TREATMENT WITH HEMIN

For HT-29 cells, the effect was mainly visible in the SRB assay. The MTT values did not change compared to the untreated conditions for concentrations of hemin lower than 50 µM (**Figure 8a**). However, the SRB values increased with more than 20% compared to the untreated conditions at hemin concentrations of 5 µM to 25 µM, implying more proteins in the wells and thus more cells. A concentration of 50 µM was cytotoxic for the HT-29 cells, as the MTT and SRB values then decreased significantly, and the LDH levels in the medium rose significantly.

For Caco-2 cells, the MTT and SRB values increased simultaneously, whereas the LDH level did not change relatively to the untreated conditions (**Figure 8b**). These observations indicate that the presence of hemin in these concentrations probably stimulated cell proliferation. The values changed with more than 20%, and therefore we consider the results as biologically relevant, as they cannot be assigned solely to natural inter-well variability. The strongest effect was visible at 5 µM hemin, whereafter the values decreased again and a toxic effect was seen, which was confirmed by a simultaneous increase in LDH levels.

The HTC 116 cells were less sensitive to the effect of hemin. The MTT and SRB values remained constant for the lower hemin concentrations, until the values dropped probably due to a toxic effect of the higher hemin concentration, as demonstrated by increasing LDH levels starting from a concentration of 10 µM (**Figure 8c**).
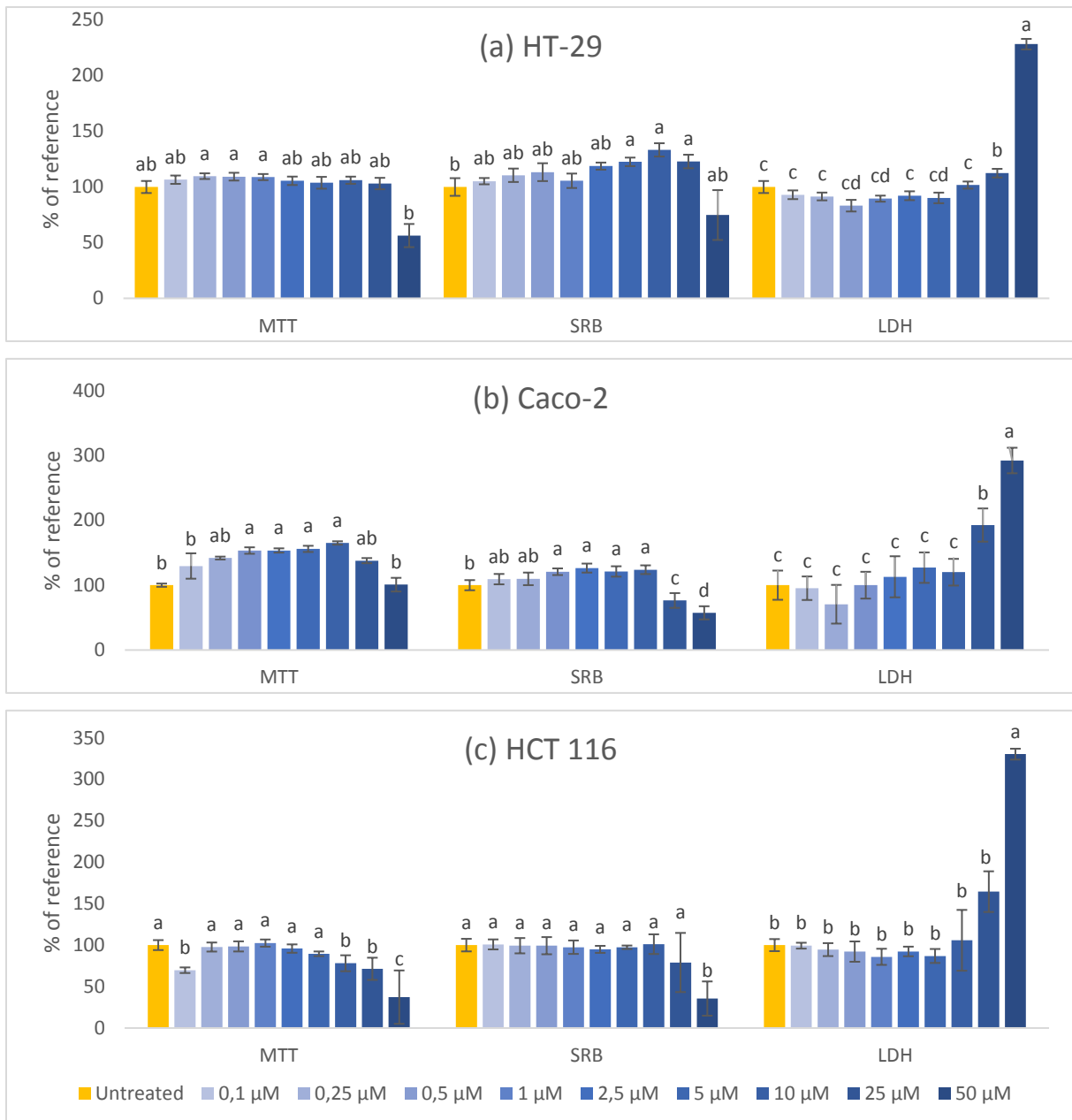
Figure 8: Results of the MTT, SRB and LDH assays for the cells treated with hemin. Mean values grouped with a different letter are significantly different from each other, based on a One Way ANOVA test (p<0.05).

The heme hypothesis is often linked to oxidative stress, and therefore, the glutathione assay was performed. However, hemin in these low concentrations did not induce significant oxidative stress to the cells (**Figure 9**). A small increasing trend in GSSG/GSH was seen for Caco-2 cells, whereas a decrease of 40% in GSSG/GSH was observed for the HT-29 cells treated with hemin compared to the untreated cells. This implies that low concentrations of hemin had a reducing effect, but no clear dose-dependent relation was observed.
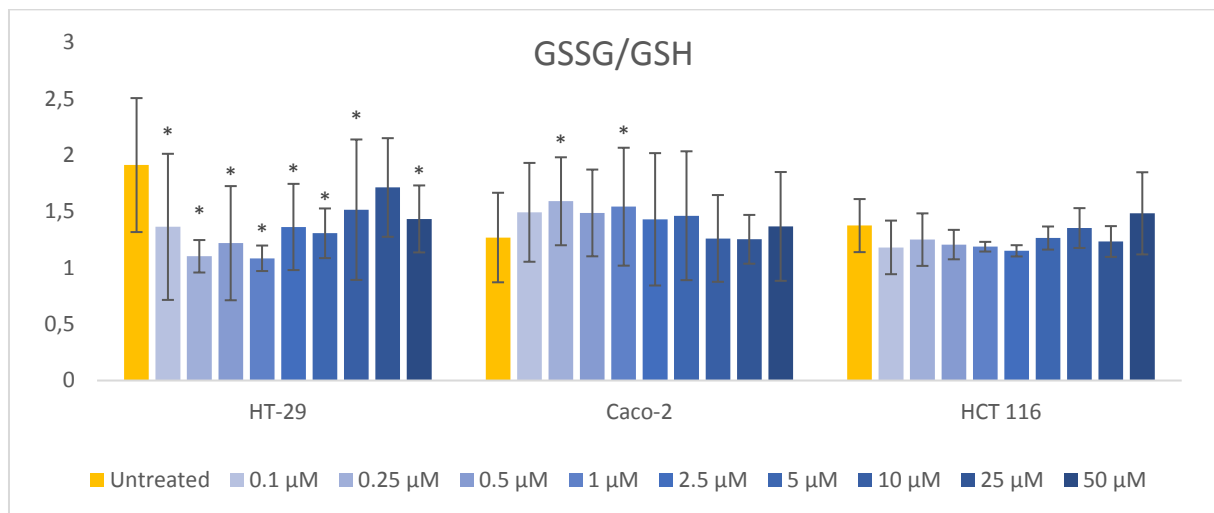

Figure 9: GSSG/GSH ratios after three days exposure to hemin. A * signifies a difference of more than 20% with the untreated cells.

An additional observation was that the hemin influenced the color of the medium, especially at the highest concentrations, turning it into a darker red, brownish solution. Moreover, an aliquot of the dilutions of hemin that were used for the cells, were kept separately in the incubator, and after three days, some hemin precipitation was seen in the most concentrated solutions (25 and 50 µM). On the contrast, no hemin crystals were found in the wells treated with these concentrations of hemin. This means that in the presence of the cells, the hemin was prevented from precipitating.

1.5 TREATMENT WITH KYNURENINE

For HT-29 and Caco-2 cells, the presence of kynurenine in different concentrations did not cause significant differences for MTT, SRB and LDH values of the treatments in relation to the untreated cells.

On the other hand, the HCT 116 cells responded to the treatment. The MTT values increased significantly, whereas SRB and LDH values did not differ in relation to the untreated cells (**Figure 10**). Thus the cells were slightly activated which caused an increase in mitochondrial activity without influencing the number of cells and without inducing a cytotoxic effect.
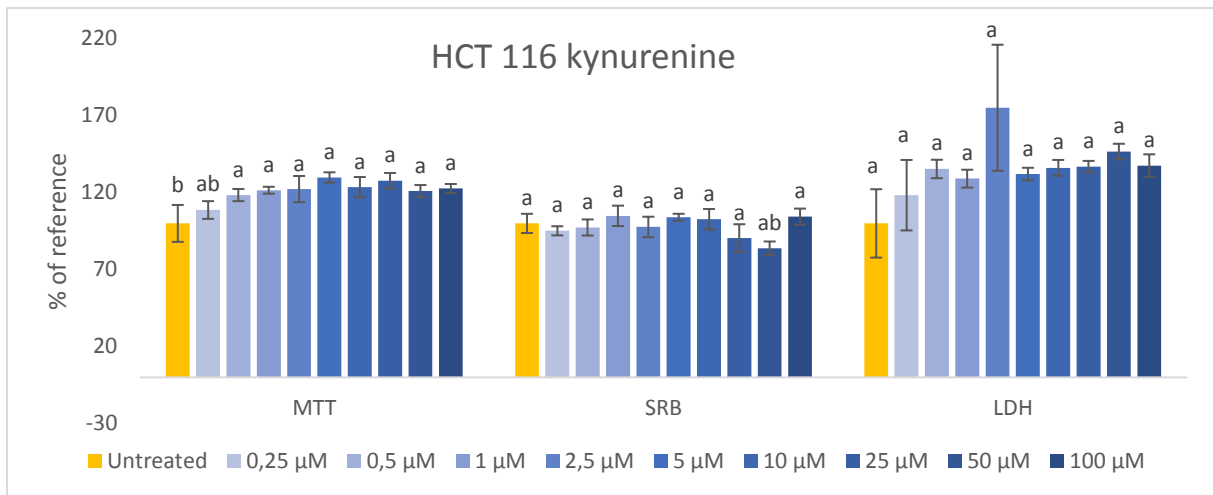
Figure 10: Results of the MTT, SRB and LDH assays for HCT 116 cells treated with kynurenine. Mean values grouped with a different letter are significantly different from each other, based on a One Way ANOVA test (p<0.05).

In contrast to the small differences on MTT, SRB and LDH assays, more pronounced changes were observed in the GSSG/GSH results (**Figure 11**). An increase in GSSG/GSH ratio was seen for Caco-2 and HCT 116 cells, compared to the untreated conditions. This implies that kynurenine induced oxidative conditions within these cells, with an effect that increased to a peak around 0.5 to 5 µM kynurenine and then decreased again. Although a similar trend was observed for the HT-29 cells, the changes were not significant.
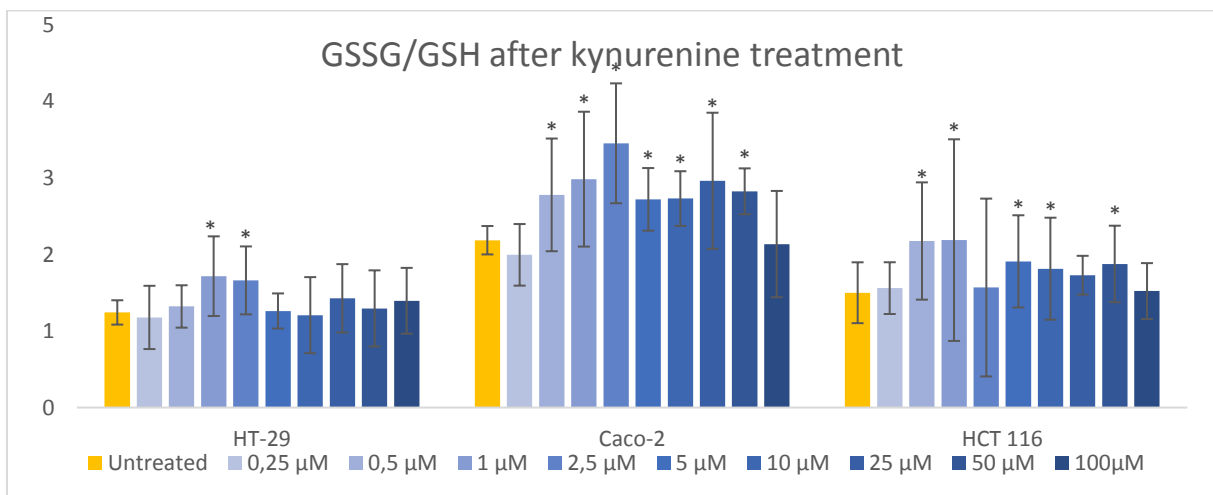

Figure 11: GSSG/GSH ratios after three days of exposure to kynurenine in different concentrations. A * signifies that the difference with the untreated cells is higher than 20%.

2 LONG TERM

2.1 OPTIMIZATION OF THE PROTOCOL

2.1.1 Calibration curves

Before the actual long-term experiments could be started up, a protocol was developed to count the cells indirectly (to avoid time-consuming counting in a Bürker chamber). A fast counting method was of importance during the long-term experiments, as we wanted to evaluate the cell growth in function of the different treatments, but we also wanted to test as many conditions as possible in parallel. The quantification method of choice was the Bradford assay, as in general, protein content is directly related to cell counts. The Bradford absorbance was determined for known numbers of cells of each cell line and combined with the established BSA-protein curve, to result in the calibration curves found in **Figure 12**. Interesting, the cellular protein content of Caco-2 cells was considerably larger than the HT-29 and HCT 116 cells, which is in line with the larger size of the Caco-2 cells compared to the other cell lines, as could be observed using microscopy.
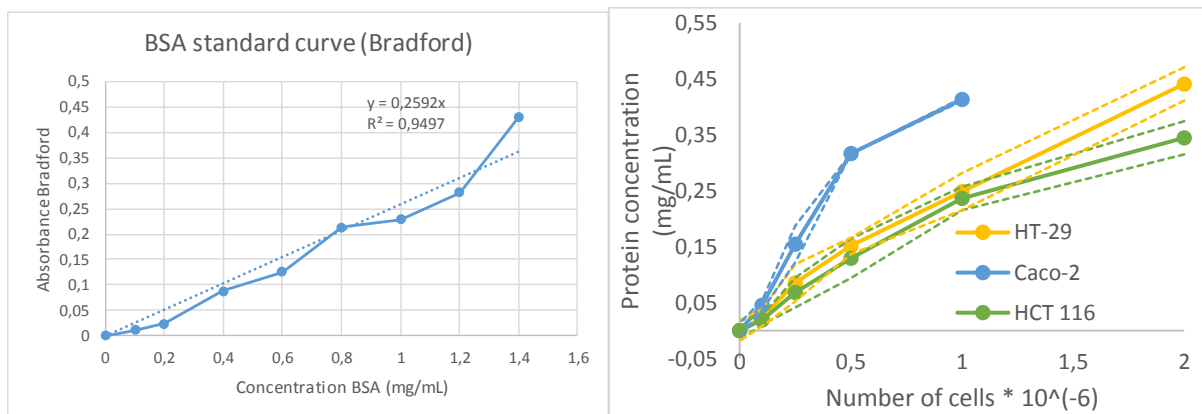

Figure 12: Absorbance from the Bradford assay in function of the concentration BSA (left) and calibration curves established with the Bradford protocol (right). The dotted line left signifies the trendline, whereas on the right, the dotted curves signify the data±S.D.

2.1.2   Optimization of lysis buffer volume

During the long-term experiment, the cells were split and counted with the Bradford procedure as described above. For the calibration curves, 500 µL and 100 µL of Triton X100 lyse buffer were used to lyse the pellet after centrifugation. In this case, high numbers of cells were available, as they were cultured in T75 flasks. Yet, to avoid the use of high amounts of medium when several experiments were performed in parallel, the use of 12 well plates, and therefore lower cell amounts, was preferred. Subsequently the volume of lyse buffer had to be reduced in order to attain an absorbance from the Bradford assay that was in the linear area of the calibration curves. To optimize this volume, a 12-well plate was seeded with HCT 116 cells in a density of 237.500 cells, and incubated until a confluency of approximately 80% was reached. The cells were washed with PBS and 300 µL of trypsin was added, and again removed after about 20 seconds. Then, six different volumes of Triton X100 lysis buffer were used to lyse the cells, including 50 µL, 100 µL, 250 µL, 500 µL, 750 µL and 1000 µL. A Bradford assay was performed on each of these lysates. The results when using 100 µL Triton X100 lysis buffer all fitted within the linear part of the calibration curves, so this volume was chosen to be used in the protocols of the long-term exposure experiments.

2.2 Long-term exposure

In contrast to the short-term tests, the solutions of the components were made with growth medium containing FBS (Fetal Bovine Serum). In the short-term tests, this was avoided as the serum may cause extra variability, because of its biological origin. On the long term however, it is not possible to do this, as the cells would struggle to grow at a normal rate without the serum.

The tested concentrations in each of the long-term experiments are summarized in **Table 4**.

Table 4: Overview of the tested concentrations in the long-term experiments

| Cell line | Component | Trial 1 (7 days incubation) | | Trial 2 (12 days incubation) | | IncuCyte® and clonogenic assay (12 days incubation) | |
		Condition	Concentration	Condition	Concentration	Condition	Concentration
Caco-2	Butyrate	1	0.5 mM	1	1 µM	1	1 µM
		2	5 mM	2	10 µM	2	10 µM
				3	50 µM	3	50 µM
						4	100 µM
	Hemin	3	0.1 µM	4	0.001 µM	5	0.001 µM
		4	1 µM	5	0.01 µM	6	0.01 µM
				6	0.1 µM	7	0.1 µM
						8	1 µM
	Kynurenine	5	0.25 µM	7	0.25 µM	9	0.1 µM
		6	1 µM	8	1 µM	10	0.25 µM
						11	1 µM
						12	2.5 µM
	Untreated	7	Medium	9	medium	13	medium
HT-29	Butyrate	8	0.5 mM	10	1 µM	14	1 µM
		9	5 mM	11	10 µM	15	10 µM
				12	50 µM	16	50 µM
						17	100 µM
	Hemin	10	0.1 µM	13	0.001 µM	18	0.001 µM
		11	1 µM	14	0.01 µM	19	0.01 µM
				15	0.1 µM	20	0.1 µM
						21	1 µM
	Kynurenine	12	0.25 µM	16	0.25 µM	22	0.1 µM
		13	1 µM	17	1 µM	23	0.25 µM
						24	1 µM
						25	2.5 µM
	Untreated	14	Medium	18	medium	26	medium
HCT 116	Butyrate	15	0.5 mM	19	1 µM	27	1 µM
		16	5 mM	20	10 µM	28	10 µM
				21	50 µM	29	50 µM
						30	100 µM
	Hemin	17	0.1 µM	22	0.001 µM	31	0.001 µM
		18	1 µM	23	0.01 µM	32	0.01 µM
				24	0.1 µM	33	0.1 µM
						34	1 µM
	Kynurenine	19	0.25 µM	25	0.25 µM	35	0.1 µM
		20	1 µM	26	1 µM	36	0.25 µM
						37	1 µM
						38	2.5 µM
	Untreated	21	Medium	27	medium	39	medium

2.2.1 First trial

The first trial was mainly meant to optimize the technique and the concentrations of the chosen components, as it was unclear which effect would be seen on a longer term. So initially, each component was added to the cells in two different concentrations, each in three-fold. The selection of the concentrations was based on the results of the short-term tests, as we chose the lowest concentration that had an effect after three days, and the highest concentration that had no effect after three days. As a reference, also three wells of every cell line were treated with normal growth medium ("untreated"). The first long-term experiment continued for 7 days. Some morphological changes were observed by checking the cells through the microscope on a regular base.

- Cells treated with 5 mM butyrate already displayed changes after two days of treatment. The medium had a darker red color than the other treatments for every cell line. After adding trypsin, the cells did not detach from the bottom and form single cells, but formed 'clumps', in contrast to the untreated cells, that looked normal. Overall, there were also a lot of cells that floated in the medium. After 7 days, the cells with the 5 mM butyrate treatment were no longer viable. Cells that were treated with 0.5 mM of butyrate also showed changes. The Caco-2 cells were more granular, irregularly shaped, and did not grow very much. HT-29 cells also formed 'clumps' of cells as a reaction to the toxicity. HCT 116 cells were less sensitive to the butyrate treatment. The hypothesis for these observations and the observations of the short-term tests with butyrate is that differentiation occurred, especially for Caco-2 and HT-29 cells. HCT 116 are known to differentiate less, which was also observed.
- HT-29 cells treated with hemin also formed these 'clumps', but here, cytotoxicity was a more plausible explanation.
- For kynurenine, the effects were less clear.

Every two days, the cells were trypsinized and split in a lower density. The amount of cells was estimated based on the Bradford results. This estimated cell number for each treatment is shown relatively to the untreated cells in **Figure 13**. The treatments with butyrate decreased the cell number significantly compared to the untreated cells after 4 and 7 days, which supports the hypothesis of differentiation, as this goes hand in hand with a growth stop. The influence of hemin of the estimated cell number was less clear, but microscopic assessment indicated that the cells showed signs of toxicity. Hence, the experiment was ended, as the cells were no longer suitable to be sub-cultured. Kynurenine had no toxic effect on the cells. Moreover, the estimated cell number increased compared to the untreated cells, sometimes after an initial drop in estimated cell number. Kynurenine thus had an activating effect on cell growth during the 7 days of exposure.
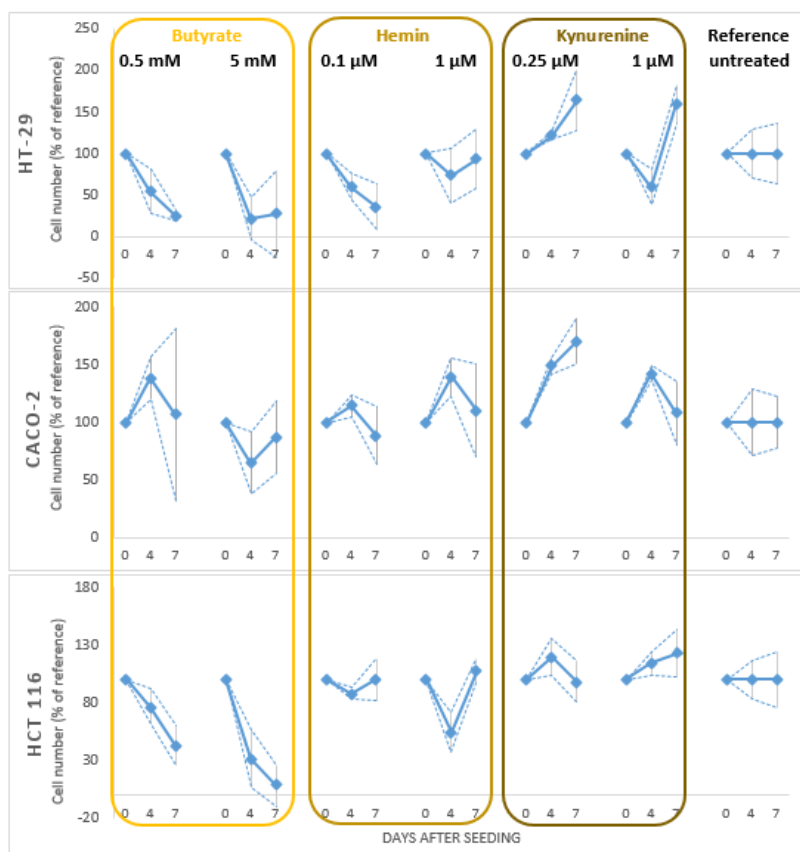
Figure 13: Estimated cell number at each time step of the first long-term exposure trial. The S.D. is indicated by an upper and lower dotted line.

No endpoint measurements were done.

2.2.2 Second trial

Considering the results of the first trial, a second long-term experiment was designed and performed, with more diluted concentrations (**Table 4**), each tested in three-fold, which lasted for 12 days. After another three days of exposure to the components, MTT, SRB, LDH and glutathione assays were performed. In addition, an aliquot of the HT-29 cells were samples for Seahorse analysis. The cells were followed up by visual assessment on a regular base. Some morphological changes were observed.

- In general, a lot of floating cells and cell debris was seen for Caco-2. Moreover, Caco-2 cells treated with butyrate had a flatter appearance with less sharp edges. Caco-2 cells with hemin formed unusual dome-shapes, which are common at confluency, but unexpected in this case. After 9 days, the medium of untreated Caco-2 cells was more red than for all of the treatments, indicating that the metabolism of these cells was less active. Many dead cells were observed for this untreated condition. The medium of HT-29 cells treated with hemin appeared more yellow. Also for kynurenine, the medium showed a dose-dependent color change: the highest concentration resulted in a more yellow medium color, for all the cell lines (**Figure 14**).

- HT-29 cells treated with butyrate also had the same, very subtle, morphological changes as Caco-2 cells. The cells seemed less contoured and formed a flat surface of cells.
- After 12 days, the Caco-2 cells lost all viability because of a contamination. No endpoint-measurements were done on these wells.

Figure 14: Pictures of the medium color of the plates from the second long-term exposure trial 2. B1: 1 µM, B2: 10 µM, B3: 50 µM, H1: 0.001 µM, H2: 0.01 µM, H3: 0.1 µM, K1: 0.25 µM, K2: 1 µM, blanco: untreated cells.

In **Figure 15,** the evolution of the estimated cell number in function of time is shown. All the results are expressed relatively to the number of untreated cells.

The presence of butyrate at these lower concentrations induced a higher cell number compared to the untreated cells. Especially the Caco-2 cells reacted strongly to the treatments, but also the HT-29 and HCT 116 responded by proliferating more than the untreated cells at a concentration of 50 µM.

Also the hemin was much more diluted, so that the toxicity seen in trial 1 was eliminated. At the lowest concentration, no significant effects were seen, but at a concentration of 0.1 µM, HT-29 cells and Caco-2 cells were much higher in cell number than the untreated cells, even up to 150% and 200% of the cell number of the untreated cells. HCT 116 cells do not respond much to the hemin, but a slight increase in cell number was also seen.

The previously observed activating effect of kynurenine was confirmed in this second long-term experiment. Again, first a drop in cell number was seen, but after a couple of days, the cells started to divide more than the untreated cells, thereby resulting in higher estimated cell numbers. Both concentrations had this effect.
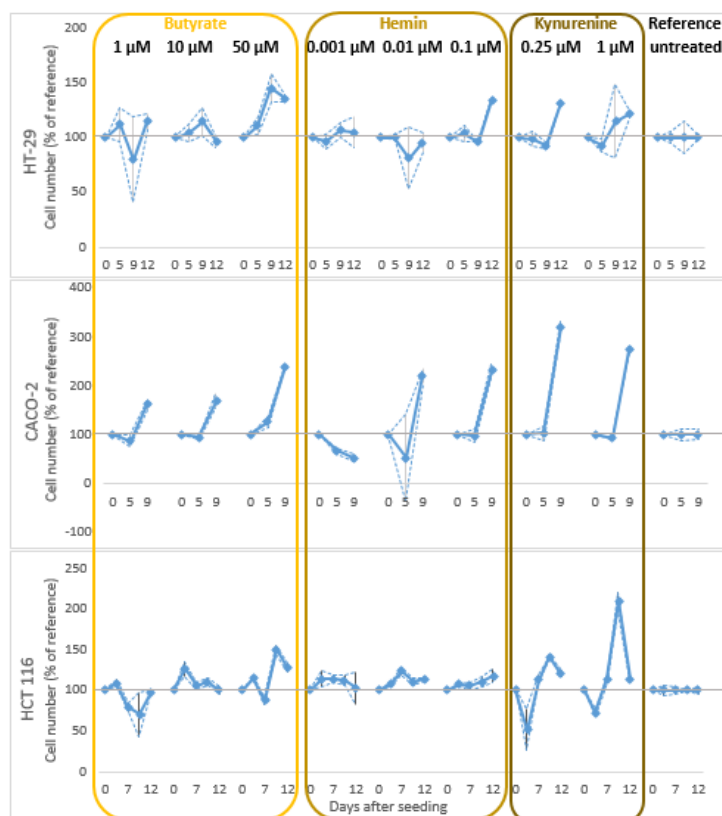
Figure 15: Estimated cell number at each time step of the second long-term exposure trial. The S.D. is indicated by an upper and lower dotted line.

For this trial, some endpoint measurements were done on the HT-29 and HCT 116 cells. The graphs can be found in the **Supplementary Data** section (**Figure 25**).

Overall, the treatments had no effect on the LDH level in the medium of HT-29 and HCT 116 cells. This means that the chosen concentrations are indeed sub-toxic. Moreover, there were no significant trends in the results, with the exception of the kynurenine treatments. Kynurenine caused the MTT and SRB values to increase to 150% and 136% of the untreated cells. This is again an indication that kynurenine may have a proliferating effect.

The most interesting endpoint assay was the glutathione assay. Although there was a high variability in the GSSG/GSH data, some trend were present (**Figure 16**). Butyrate treatments caused the GSSG/GSH ratio of HT-29 cells to decrease in a dose-dependent way. At a concentration of 50 µM butyrate, the ratio is only 50% compared to ratio of the untreated cells, indicating a lower oxidative stress. This effect is less visible in HCT 116 cells. In contrast, HCT 116 cells experience a dose-dependent lower oxidative stress when treated with hemin. HT-29 cells were also influenced by hemin, but there, 0.1 µM hemin increased the GSSG/GSH ratio again compared to the treatment with 0.01 µM hemin, which decreased

the ratio in a similar way as was seen for the HCT 116 cells. Cells treated with 0.25 µM kynurenine experienced a lower oxidative stress, whereas 1 µM kynurenine did not influence the ratio.
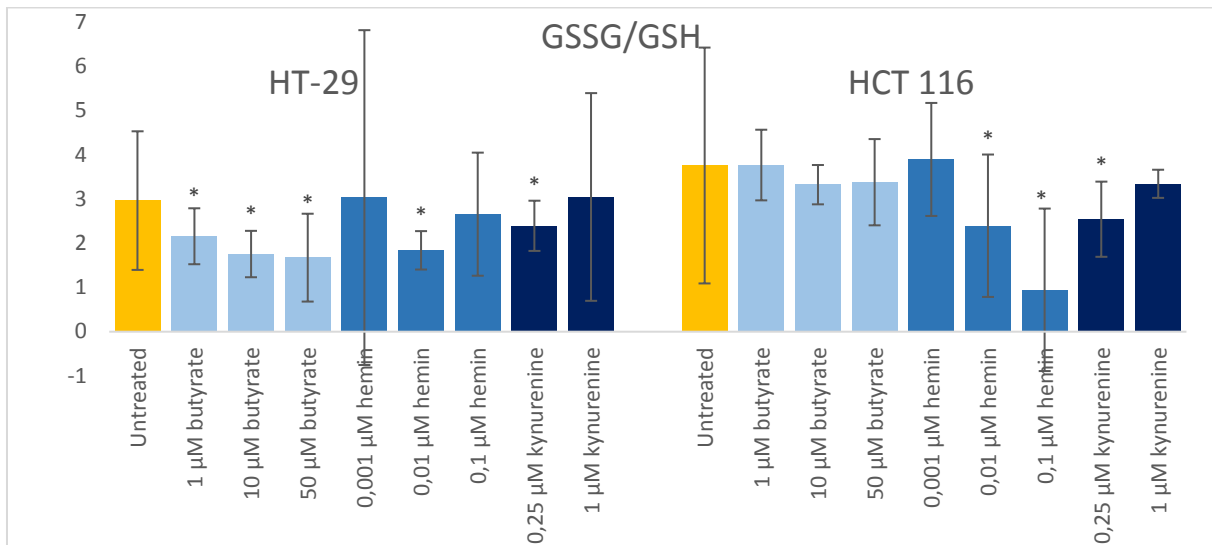

Figure 16: GSSG/GSH ratios after 15 days of exposure. A * signifies that the difference with the untreated cells is higher than 20%.

2.3 SEAHORSE ASSAY

The results of the OCR and ECAR of both technical replicates is presented in **Figure 17**. A first observation is that these values vary amongst the first and second replicate with 5 hours of difference. This can be explained by the fact that with the first replicate, the cells just started to grow, and were not well stretched out over the well surface yet, as was assessed using microscopy. During the second repetition, the cells were clearly in their growing phase, and therefore, this second repetition is considered to be the most representative one, although some trends were visible in both repetitions.

A second observation is the differences in variability amongst the different treatments. Whereas the standard deviations of the OCR in untreated and butyrate treated cells were relatively acceptable, they tend to increase in some of the hemin and kynurenine treated cells. This may indicate that the continuous exposure of the cells to these agents may induce a wider diversity in cell phenotype.

A third observation was that butyrate clearly increased basal and total OCR at all tested concentrations compared to the untreated cells, whereas a differential effect for the other compounds was seen. For butyrate, this was combined with high ECAR, which may point to the action of energy production through both mitochondrial as non-mitochondrial pathways. In addition, although the addition of glucose only counted for a small increase in OCR, the effect of oligomycin substantially increased the OCR compared to the untreated cells.

For hemin treatment, if we take into account that 1 μM (H4) was toxic for the cells and therefore only consider the sub-toxic effects, we see a trend towards a lower OCR and increased ECAR, at the basal level and after glucose addition, with increasing hemin concentration. For the toxic concentration, the higher OCR may point to a stress response, and the variability may be explained by the existence of cells with very high mitochondrial activity, and the dead cells with very low activity.

For kynurenine, the trends are more difficult to interpret in a dose-response way. If we consider the second replicate, the two lowest concentrations resulted in OCR profiles similar to the untreated cells, although the ECAR value of treatment with 0.25 μM kynurenine (K2) strongly increased. This increasing trend was also visible for treatment with 1 μM kynurenine (K3) although here the spare mitochondrial respiration after antimycin/rotenone shut-down was lower. For the highest concentrations, the OCR and ECAR were very low, although the cells were clearly growing, as confirmed by similar SRB value increases as for the untreated cells, and microscopic evaluation. Therefore, energy production in the cells was probably the results of other mechanisms than mitochondrial respiration and anaerobic glucose metabolism.
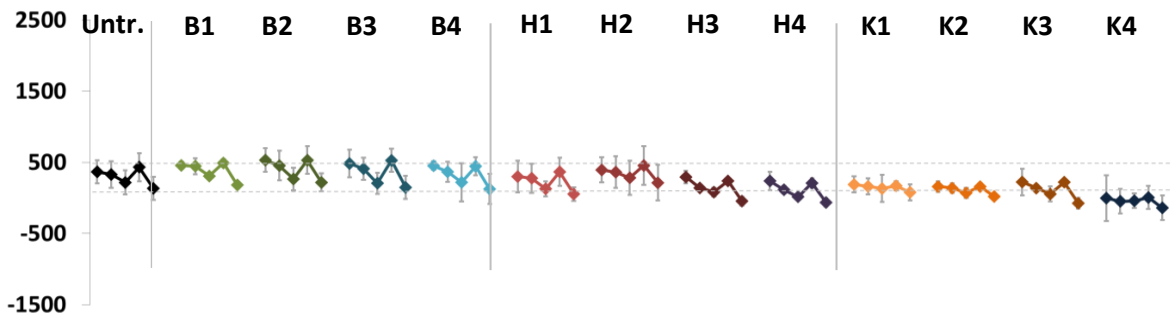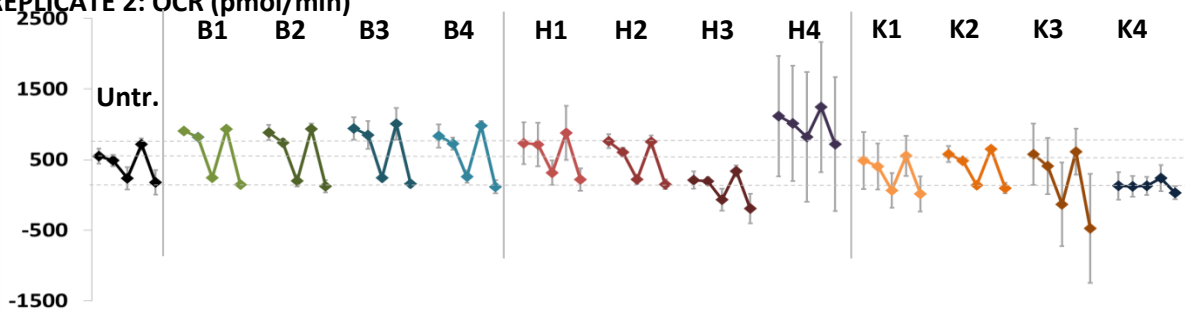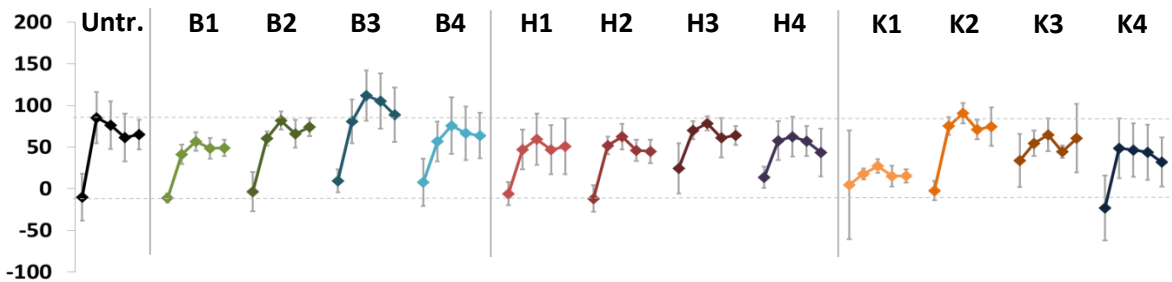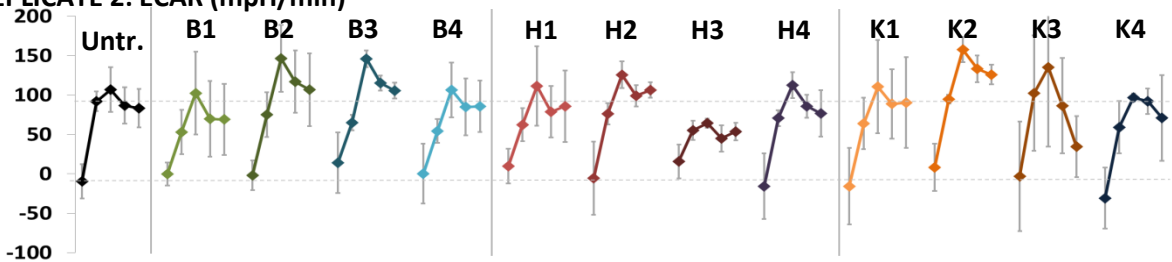
Figure 17: SRB-corrected oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of the long-term pretreated HT-29 cells (3 biological replicates) with butyrate, hemin and kynurenine in 4 concentrations: Untr.: untreated cells, B1: 1 µM butyrate, B2: 10 µM butyrate, B2: 50 µM butyrate, B4: 100 µM butyrate, H1: 0.001 µM hemin, H2: 0.01 µM hemin, H3: 0.1 µM hemin, H4: 1 µM hemin, K1: 0.1 µM kynurenine, K2: 0.25 µM kynurenine, K3: 1 µM kynurenine, K4: 2.5 µM kynurenine. The first measurement point is the average basal respiration, without addition of glucose. The second time point reflects the response after glucose addition, and the following three time are measurements after oligomycin, FCCP and antimycin/rotenone, respectively. The results are displayed as the average of the 3 time points after each injection of the 3 biological replicates (n=9), and error bars indicate standard deviations. Replicate 1 and 2 are technical replicates with approximately 5 hours of time difference.

2.4 INCUCYTE® LIVE CELL ANALYSIS

The IncuCyte® is a powerful tool to estimate cell number per well. The cells were seeded in a very low density (4000 cells per well on a 96-well plate) **(Supplementary data, Figure 26)**, so that they did not reach confluency within the course of the experiment, thereby eliminating the need of a splitting step. Hence, the possible interfering effects of the components on cell adhesion were excluded.

The IncuCyte experiment was performed with HT-29 cells from the LECR laboratory. The choice for this cell line was based on the results of the previous exposure experiments, but mostly on the intermediate characteristics of these cells. In such a low seeding density, Caco-2 cells would probably struggle to get launched for growth, and it would take too long before the effects are perceptible. On the other hand, HCT 116 cells growth very fast and would reach confluency before the end of the experiment. HT-29 cells are the ideal compromise.

Using the IncuCyte software, a 'masking' is performed on the images, which means that the area on the picture that should be defined as 'cells' was determined. The program could then use this masking on all the other pictures in order to calculate how much of the surface of each well is overgrown with cells, which is expressed as a percentage of confluency. A validation step was performed to exclude edge effects and to confirm if the reference wells gave reliable results, as is demonstrated in the **Supplementary Data (Figure 27)**.

The data can be visualized by generating one curve per well, showing the confluency as the percentage of well surface that is covered by cells in function of time (**Supplementary data, Figure 28**), or as a mean percentage of confluency per condition (**Figure 18, 19 and 20**). For the butyrate and hemin treatments, the experiment continued on for 12 days (=288 hours). For the kynurenine plate, the experiment was ended after 9 days (216 hours). At seeding, the curves coincide, as the same number of cells was present in each well and each treatment. After a few hours, the treatments caused differences in growth rate. The jumps in the curve were caused by adding fresh medium, triggering an immediate reaction of the cells.

The butyrate treatments of 1 µM, 10 µM and 50 µM induced a higher average percentage of confluency than the untreated cells, whereas the highest concentration of 100 µM butyrate did not have a significant effect. However, a trend could be observed which was in line with previous observations, as lower concentrations of butyrate stimulated cell growth, whereas at higher concentrations, this effect disappeared and an opposite effect was seen. This may indicate that the concentration of butyrate had to be higher in order to see the growth inhibition (differentiating) effect that was seen in the short-term experiments.

The highest concentration of hemin (1 μM) was clearly toxic for the cells, as was seen in the previous experiments. The other treatments induced a slight increase in cell number in comparison to the untreated condition. The strongest proliferative effect was seen for the curves of 0.001 μM and 0.01 μM hemin, followed by the 0.1 μM-curve. Lower concentrations thus induced a proliferative effect, but the effect disappeared as the hemin concentration increased, most probably because of toxicity, as was seen in the short-term experiments.

The results for the kynurenine treatments were more variable. The lowest concentrations of kynurenine (0.1 μM and 0.25 μM) induced a higher percentage of confluency than the untreated cells. At higher concentrations (1 μM and 2.5 μM) this effect was not so pronounced.
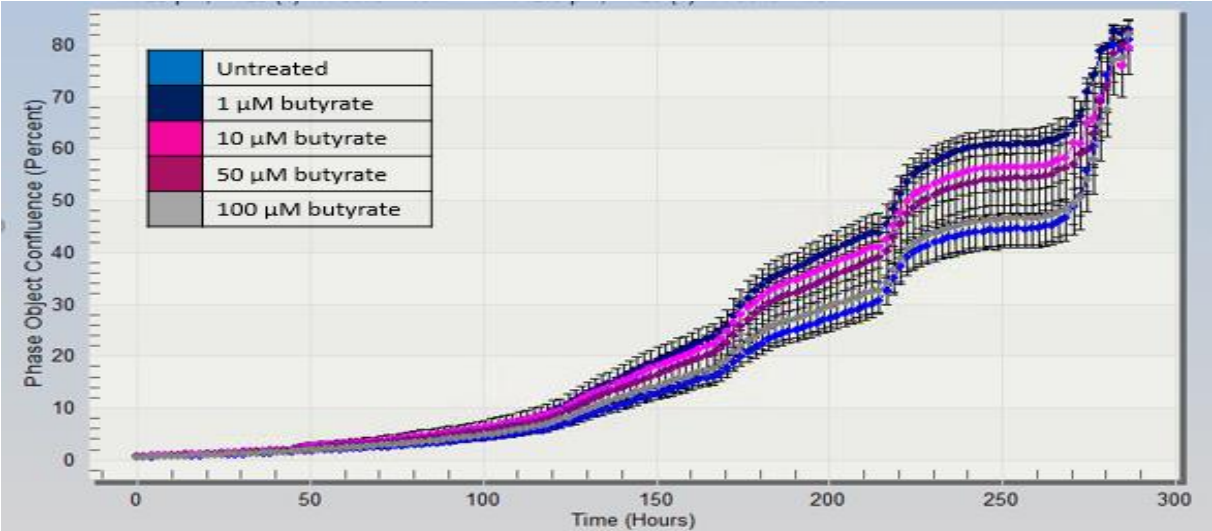
Figure 18: Mean confluency (% of the well surface) of butyrate treatments in function of time.
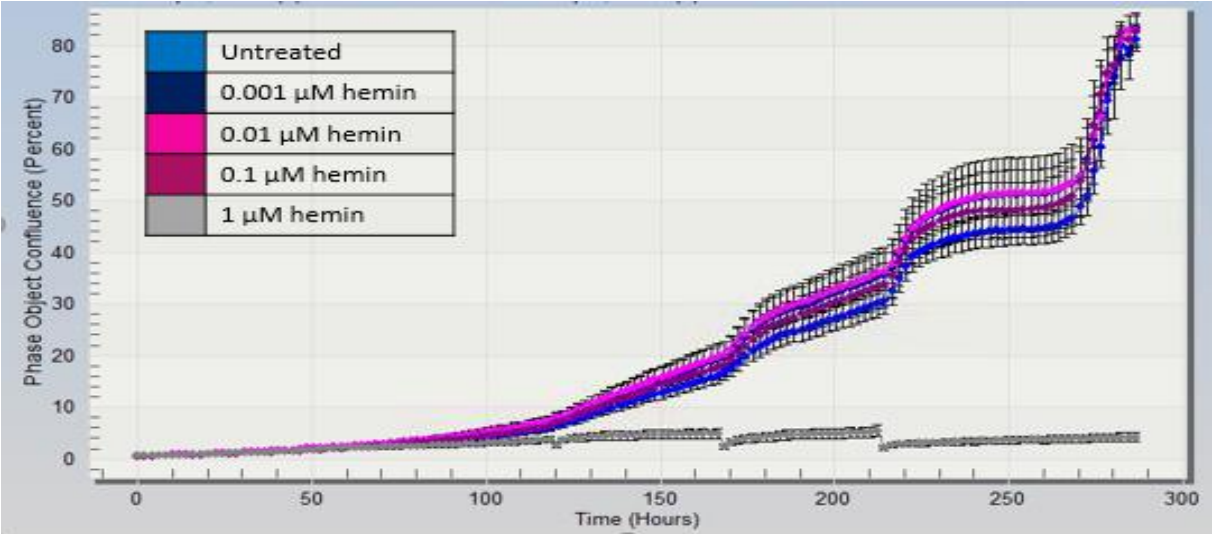
Figure 19: Mean confluency (% of the well surface) of hemin treatments in function of time.
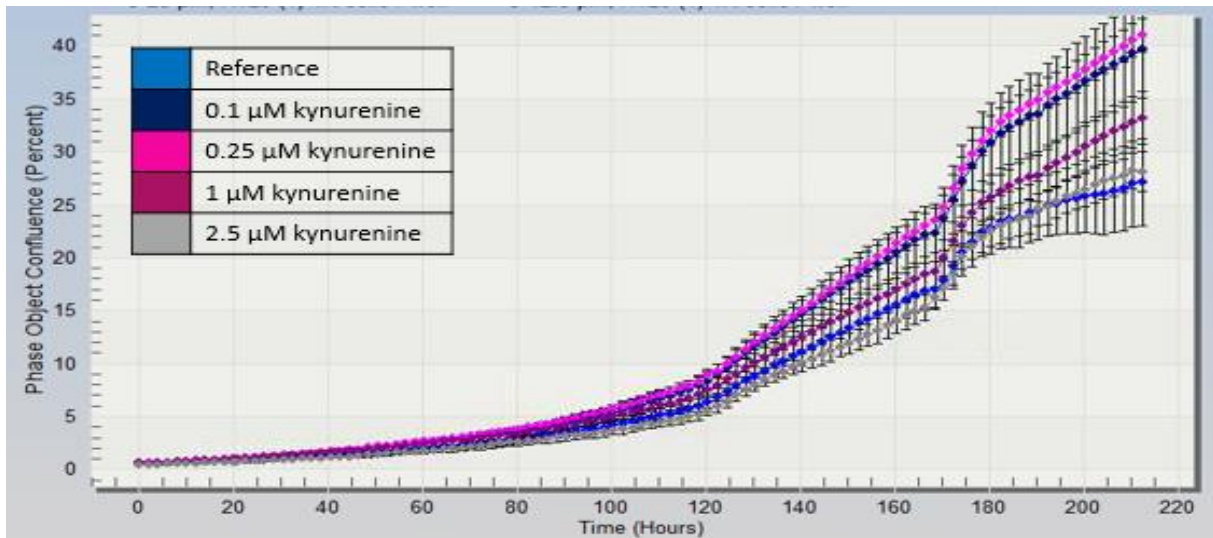
Figure 20: Mean confluency (% of the well surface) of kynurenine treatments in function of time.

A parameter that can be derived from the data is the doubling time for the HT-29 cells. This numeric value is then an indication of how the treatment influences the growth of the cells. The time that the cells need to go from a confluency of 10% to a confluency of 20%, as calculated by the IncuCyte Software, gave an idea of the doubling time. The exact values are shown in the **Supplementary data, Table 7**. The same trends as described above were observed.

2.5 CLONOGENIC ASSAY

In parallel with the IncuCyte experiment, a clonogenic assay was performed on the same batch of HT-29 cells.

The results of the clonogenic assay are shown in **Figure 21**. Between 20 and 30% of the seeded cells eventually formed colonies, with exception of those treated with 1 µM hemin (6%). No biologically relevant differences were present for the butyrate treatment. The hemin does induce some effects, similar to the results discussed before. The 0.01 µM hemin treatment resulted in a higher number of colonies, whereas the highest concentration was clearly toxic. Kynurenine seems to increase the number of colonies at the lowest concentrations, but didn't have a significant impact for the other concentrations. However, the colonies were visibly smaller and less dense than the untreated condition at the highest concentrations (1 and 2.5 µM). The components thus probably affected the number of cells per colony rather than the number of colonies.
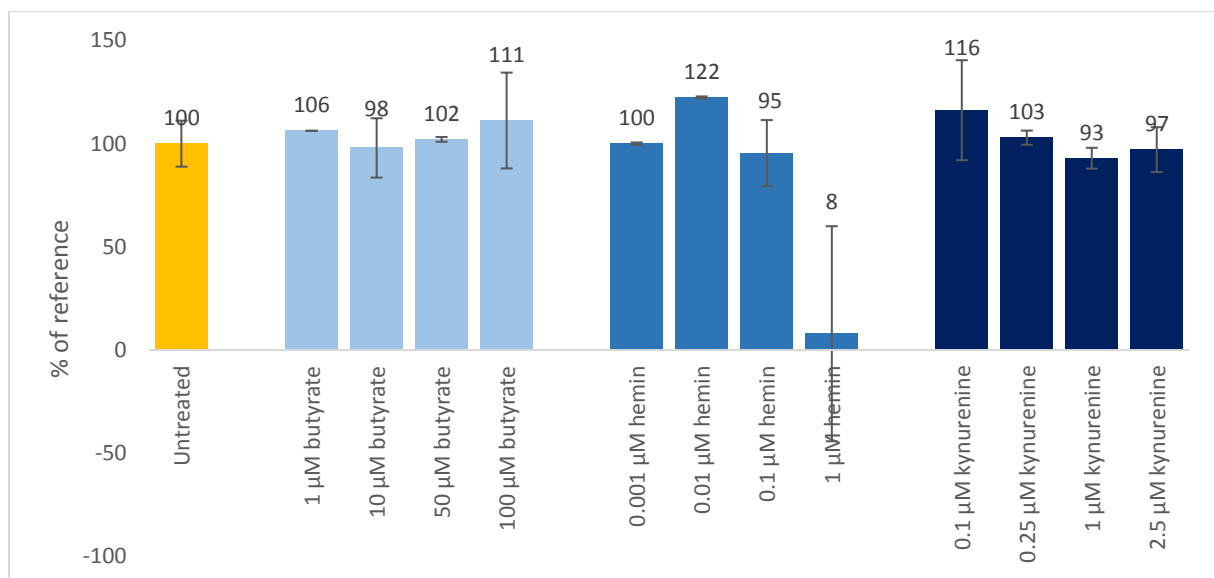

Figure 21: Results of the clonogenic assay: number of colonies per treatment expressed as a percentage of the untreated cells.

Discussion

1 INTRODUCTION

Colon cancer development is a slow process that can take more than 15 years. Therefore, we consider the long-term exposure of intestinal cells as a relevant topic that has been understudied so far. In the intestine, stem cells in the crypts continuously proliferate to allow the intestinal epithelium to renew every 4 to 5 days (Clevers, 2013). The earliest tissue change that has been identified within the development of CRC is a proliferative shift in normal-appearing colonic crypt cells, which is associated with the APC mutation (Tao Zhang et al., 2001). This results in a population of highly adaptable and proliferative cells that have stem cell characteristics. They persist for the lifetime while producing other cell types of the epithelium (Clevers, 2013). Hence, mutations in these crypt cells have much more impact than in mature enterocytes in the development of colon cancer (Blanpain et al., 2011; Rossi et al., 2008). In addition, besides the occurrence of mutations, the micro-environment of tumor cells is now believed to play a role also in the later stages of cancer development, and the presence of certain molecules could contribute to their adaptation to chemical and physical signals, and finally to the phenotype of the cancer cells (Palmieri et al., 2017).

In this work, a novel long-term approach was used to explore the impact of sub-toxic exposure of three different intestinal cancer cell lines to meat components and microbial metabolites formed after the consumption of (processed) meat meals. **Table 5** gives an overview of the observed effects throughout the different experiments. A difference of more than ±20% compared to the untreated condition was considered as biologically relevant, as changes within this range could be due to natural variability. Effects on oxidative stress, cell proliferation and energy metabolism, rather than on cell adhesion, were observed, and these effects were compound and cell line specific. In this discussion, we highlight some of these aspects.

Table 5: Summary of the observed effects of both short and long-term experiments. The symbol '=' is used when the values did not change more than 20% of the untreated cells. '↑' stands for an increase in average value of more than 20%, whereas '↓' signifies a decrease in the average values of 20% or more in relation to the untreated condition. When different effects were seen for different concentrations, the concentration range is added. Grey zones mean that the test was not performed for those conditions. Arrows between brackets indicate that the test is probably not reliable because of technical problems.

Short term

		MTT	Conc. Range	SRB	Conc. range	LDH	Conc. range	GSSG/GSH	Conc. Range
HT-29	+ Nitrite	=		=		=			
	+ Hemoglobin	=		=		=			
	+ Butyrate	↓		↓		=			
	+ Hemin	= / ↓	0.1 → 25 µM / 50 µM	= / ↑ / ↓	0.1 → 2.5 µM / 5 → 25 µM / 50 µM	= / ↑	0.12 → 5 µM / 50 µM	↓	
	+ Kynurenine	=		=		=		=	
Caco-2	+ Nitrite	=		=		=			
	+ Hemoglobin	=		=		=			
	+ Butyrate	↓		↓		=			
	+ Hemin	↑ / =	0.1 → 25 µM / 50 µM	↑ / ↓	0.1 → 10 µM / 25 → 50 µM	= / ↑	0.1 → 1 µM / 5 → 50 µM	↑ / =	0.1 → 1 µM / 2.5 → 50 µM
	+ Kynurenine	=		=		=		↑	
HCT 116	+ Nitrite	=		=		=			
	+ Hemoglobin	=		=		=			
	+ Butyrate	↓		↓		↑			
	+ Hemin	= / ↓	0.1 → 5 µM / 10 → 50 µM	= / ↓	0.1 → 10 µM / 25 → 50 µM	= / ↑	0.1 → 10 µM / 25 → 50 µM	=	
	+ Kynurenine	↑		=		↑		↑	

Long term

		Cell number (% of ref.)	Incu-Cyte (% of confluency)	MTT	Conc.	SRB	Conc.	LDH	GSSG/GSH
HT-29	+ Butyrate	= ↑	↑=	↑ / ↓	10 µM / 50 µM	↑ / ↓	10 µM / 50 µM	=	↓
	+ Hemin	= ↑	↑=	=		=		=	↓
	+ Kynurenine	↓↑	↑=	↑		↑		=	=
Caco-2	+ Butyrate	↑							
	+ Hemin	↓↑							
	+ Kynurenine	= ↑							
HCT 116	+ Butyrate	= ↑		(↓)		↑	10 µM	=	=
	+ Hemin	=		(↓)		↑	0.1 µM	=	↓
	+ Kynurenine	↓↑		(↓)		=		=	↓

50

2 COMPOUND SPECIFIC EFFECTS

2.1 NITRITE

Treatment of the cells with pure nitrite did not affect the cell behavior when considering mitochondrial activity, cell number or free LDH levels, not even at very high concentrations. Nitrite is used as a preserving agent in meat, but in reality, only a small portion of nitrite actually reaches the colon, as it is converted into a variety of derivatives during the digestion, such as the carcinogenic NOCs. These genotoxic compounds are believed to be one of the main factors why processed meat increases the risk on CRC even more than red meat. A recent study of Etemadi et al. (2017) indeed confirmed the association between nitrate and nitrite intake through processed meat and an increased mortality, and also suggests that the mechanism involves the formation of NOCs.

Our results indicate that, at least on the short term, nitrite as such did not influence cell metabolism, not even at concentrations of 10 mM, which is far from the realistic luminal concentrations in the gut. This is in line with the conclusions of a cohort study performed by Knekt et al. (1999), who found no significant association between nitrite or nitrate intake and the risk of CRC. As far as we know, no *in vitro* studies with nitrite addition to cell lines were published before. In addition, some studies show a preventive and therapeutic effect of nitrite on specific intestinal diseases (Bryan et al., 2007; Jädert et al., 2014), so nitrite in its pure form could offer advantages for the patient, but only when it would be possible to reach the colon without conversions. To study the impact on CRC of processed meat, it would be more relevant to add nitrite digestion metabolites instead.

2.2 HEMOGLOBIN AND HEMIN

One component of red meat that is often linked to CRC is heme iron, which is present in red meat as part of myoglobin. Heme absorption in the gastro-intestinal tract is low, because of the strict regulation of iron uptake, hence most ingested heme is delivered to the colon (Conrad & Umbreit, 2000; Jeyakumar et al., 2016; Sesink et al., 1999). The heme iron has a direct and indirect effect: it is directly cytotoxic and genotoxic for epithelial cells and indirectly catalyzes the formation of other compounds that promote carcinogenesis (Bastide et al., 2015). Three potential mechanisms have been suggested before (Bastide et al., 2015; Sesink et al., 1999), but within the scope of this project, the cytotoxicity of heme is the most interesting mechanism, as we focus on cancer progression rather than cancer initiation. Nevertheless, especially the long-term exposure to hemin resulted in high variability within the samples of the same condition (Seahorse, oxidative stress), which could be a consequence of the joint effects of hemin on the cells.

To test the effect of heme, the cell lines were first treated with hemoglobin, a molecule that contains four heme groups. Hemoglobin acts as a suitable substitute for myoglobin in studies on the cytotoxicity of red meat (F. Pierre et al., 2003). After three days of treatment with a wide range of concentrations, none of the cell lines responded significantly to the treatments. The lack of an effect is probably due to the structure of the molecule, as the heme groups in hemoglobin are embedded in the protein-structure, and therefore protected from absorption. The cells probably could not hydrolyze the surrounding proteins in a sufficient way to release the heme groups as such. This structural problem was eliminated by using hemin instead of hemoglobin for new experiments. Hemin is also often used for experimental studies (Sesink et al., 2001). It is a chemical derivative of hemoglobin (Demeyer et al., 2016), in which the heme group is more available, allowing it to act more effectively on the cells. For example in a study of Glei et al. (2006), it was shown that more iron was available from hemin than from hemoglobin, and a study of F. Pierre et al. (2003) concluded that hemin was more potent in promoting carcinogenesis than hemoglobin.

The treatments with hemin indeed influenced some cellular parameters. Especially Caco-2 cells were sensitive, showing a proliferating effect both for short-term exposure as long-term exposure at low concentrations, and toxicity and cell death at higher concentrations. For this cell line, hemin stimulated the mitochondrial activity, the number of cells, and raised the oxidative stress in the cells on the short term, proportionally to the concentration of hemin. When toxicity occurred, the LDH levels raised and unusual dome-shapes were formed as a reaction to the unwanted component. The HT-29 and HCT 116 cells did not change with regard to the tested parameters on the short-term except for toxicity at the highest concentrations, and also for the long-term exposure, the effects were smaller, with exception of an increase of 50% in HT-29 cell number at a concentration of 0.1 µM after 12 days of exposure, and an increase in HCT 116 cells after 15 days. This indicates cell-line specific responses to hemin. Another observation is found in the Seahorse assay results (HT-29 cells). The lower OCR and increased ECAR at the basal level and after glucose addition point to an increased use of glucose for energy production, which could be a consequence of the Warburg effect. Also the more yellow appearance of the medium of Caco-2 and HT-29 cells exposed to hemin could be evidence of this shift from ATP generation through oxidative phosphorylation to ATP generation through glycolysis, which increases the glucose demand and lactate production, and confirms the carcinogenic phenotype of cells. As the effect is dose-dependent, this is another indication of the cancer-promoting effect of hemin. The cell lines originate from different stages of CRC, and go from earlier stages to later stages in the order of Caco-2<HT-29<HCT 116. Overall, the trends diminished as the cells originate from later stages, and therefore, we may carefully conclude that the effect of hemin is more pronounced in the cell phenotype at earlier stages in CRC progression (See also: 3. cell line specific effects).

The increased proliferation, oxidative stress, and decreased efficiency of mitochondrial respiration, comply with literature describing one of the effects of heme as 'cytotoxicity'. Heme iron triggers a toxic environment that damages the epithelium cells (IJssennagger, Rijnierse, et al., 2012; Ijsennagger et al., 2013; Sesink et al., 1999). The body tries to compensate for the damaged surface cells, so hyperproliferation of the stem cells lying in the crypts of the epithelial layer is initiated (de Vogel et al., 2008), which eventually leads to an increased amount of organ tissue, especially when also apoptosis is repressed (which is a hallmark of cancer cells). Hyperproliferation is a known risk marker of colon cancer, as it increases the risk of a malignancy (Kinzler & Vogelstein, 1996; Lipkin, 1988).

Although heme ingestion is also known to cause an acute oxidative stress and an increase in intracellular reactive oxygen species (ROS) leading to increased levels of lipid peroxidation products (IJssennagger, de Wit, et al., 2012), ROS have no causal role in the above mentioned mechanism. That was concluded from studies with C57Bl6/J mice that were fed with a Westernized purified control diet (40 en% fat , low calcium), or the same diet supplemented with either 0.2 or 0.5 µmol heme/g diet for 14 days (Ijssennagger et al., 2013). The increase in ROS in the fecal water of the mice was acute, that is, within the first two days of heme feeding. In contrast, there was a lag time before the cytotoxicity in the colonic lumen occurred. Cytotoxicity increased after day 4 and was significantly higher with heme treatment compared to the controls on days 7 and 14 (Ijssennagger et al., 2013). The proliferation increased in parallel with the cytotoxicity. This delay of the proliferative effect is similar to our results, as significant proliferation only arose after a long-term exposure and not after three days of treatment for HT-29 and HCT 116. Moreover, the GSSG/GSH data of HT-29 and HCT 116 cells did indeed show a lower or equal ratio, so the hemin did not induce ROS for the tested conditions. That observation strengthens the assumption that hemin had a hyperproliferating effect, with cytotoxicity and not ROS as causal factor, as was also observed in the heme-fed mice (Ijssennagger et al., 2013).

The hyperproliferation is a consequence of a downregulation of feedback inhibitors of proliferation, such as the Wnt inhibitory factor 1 (Wif1) and the Indian Hedgehog (Ihh) (IJssennagger, Rijnierse, et al., 2012). The Wif1 normally forms complexes with Wnts, subsequently blocking the binding of Wnts to their receptor. In the mice on the heme diet, Wif1 was downregulated fivefold (IJssennagger, Rijnierse, et al., 2012). Ihh is an antagonist of the Wnt signaling in the colonic epithelial cell differentiation process (van den Brink et al., 2004). These inhibitors are thus downregulated by the presence of hemin, thereby stimulating the Wnt signaling pathway, which is a hallmark of cancer cells. This implies that the carcinogenic effect heme could be explained partially by its impact on the signaling pathways and subsequently the metabolism of the epithelium cells.

The Wnt signaling pathway is apparently strongly involved when discussing the influence of hemin on colon cancer development. It was also already mentioned that this pathway is a typical property of stemness of cells, and it was previously reported to be influenced by the micro-environment of the cells (Vermeulen et al., 2010). The Wnt pathway should thus definitely be further assessed in the future, and especially Caco-2 cells, which still have a large potential to differentiate and are the least cancerous of the tested cell types, may be the most suitable to test this hypothesis in the future.

2.3 BUTYRATE

Butyrate is one of the short chain fatty acids (SCFAs), which are present in the colon lumen in high concentration (between 100 and 240 mM, (Barnard & Warwick, 1993)), as a consequence of bacterial fermentation of undigested dietary fibers in the colon. Diets poor in dietary fibers are linked to an increased incidence of inflammatory diseases and intestinal cancers in the developed parts of the world (Sivaprakasam et al., 2016). The three most abundant SCFAs are acetate, propionate and butyrate and are found in a ratio of 60:20:20 (Bergman, 1990; Cummings et al., 1987; Hijova & Chmelarova, 2007). Butyrate has been shown to have the most significant effects of the SCFAs on colonic epithelial cells, as well *in vivo* as *in vitro* (Hague et al., 1997). This is also demonstrated by measuring the blood concentration of butyrate in sheep, which was only 10% of that of the lumen concentration, implying that most of it is used by the colonic epithelium itself (Bergman, 1990). Physiologically realistic levels of butyrate in the colonic lumen range from 2 to 4 mM (Hague et al., 1996).

Butyrate has been studied before as a component that could possibly play a role in the maintenance of a healthy colon epithelium and subsequently has been investigated for its possible role in treatment of colon diseases. Studies have suggested that *in vivo*, SFCAs deliver the main source of metabolic energy for the colonocytes (Ahmad et al., 2000; Louis et al., 2014). Nevertheless, this effect disappears for cells of cancer origin that are cultured *in vitro*, where butyrate treatments on colon cancer cells stops cellular growth and induces cell differentiation, and even results in apoptosis (Augeron & Laboisse, 1984; Barnard & Warwick, 1993; Hague et al., 1996; Yu et al., 2010). This is called the *butyrate paradox*.

Also in our results, this paradox is present. The butyrate treatments in the short-term exposure tests were not toxic as no LDH increase was visible, but caused a growth inhibition. The same was observed in the first trial of long-term exposure, where the concentrations were similar to the short-term tests. In contrast, in much lower concentrations and upon longer exposure (trial 2), the cells increased in number compared to untreated cells. This proliferative effect increased with time, implying that the longer the exposure, the better the cells were able to cope with the presence of the butyrate, most probably even using it in their pathways.

The exposure to higher concentrations most likely made the butyrate level exceed the metabolic capacity of the cells, causing the butyrate to accumulate and overshoot the minimum threshold of activity, allowing it to act as a differentiating factor (Bultman, 2014; Donohoe et al., 2012). The hypothesis of differentiation is justified by the observed growth-inhibition and moreover by the flatter appearance and less sharp edges of the cells, which point to a more differentiated profile and a better OCR, as was also measured in the Seahorse assay. The same dual effect happens in vivo, where a concentration gradient of butyrate is present along the villi (**Figure 22**).
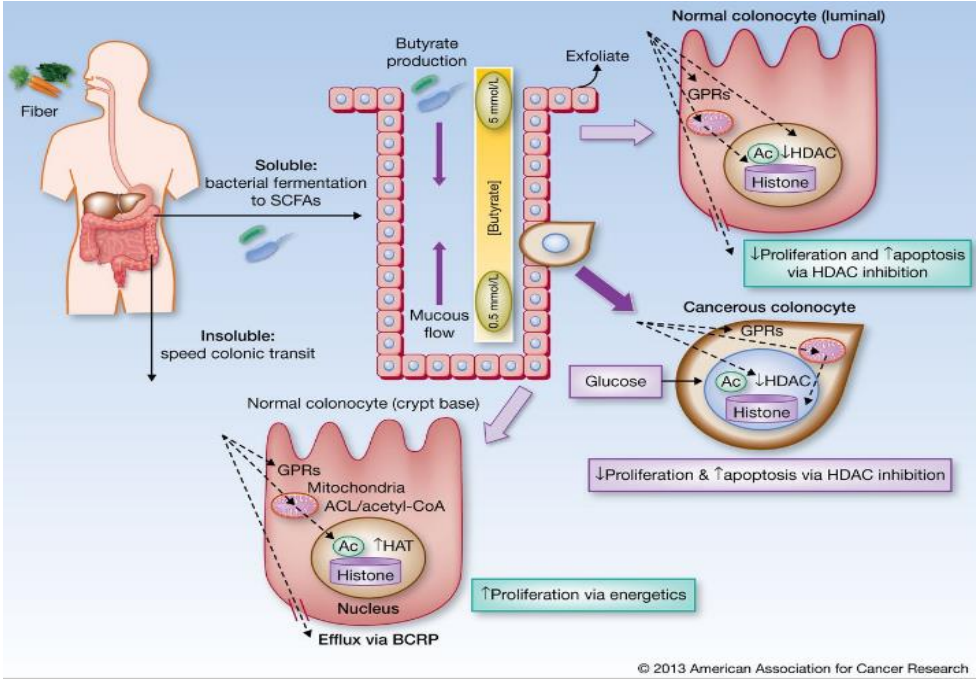

Figure 22: Schematic overview of the concentration-dependent effects of butyrate on colon(cancer)cells (Bultman, 2014).

Cellular differentiation is a complex phenomenon, characterized by the interruption of the cell cycle and the transition to alternative pathways, resulting in a more sophisticated phenotype and function. An example of a normal differentiation process *in vivo* is the differentiation of epithelial cells of the small intestine and the colon as they migrate further away from the proliferative zone in the crypts of the villi or the colon glands. The inhibitory effect *in vitro* and the stimulatory effect *in vivo* suggest that the *in vivo* effect of SCFA must be indirect (Bergman, 1990).

It is nowadays believed that accumulated butyrate enters the nucleus and targets histone deacetylases (HDAs) (Bultman, 2014; Thangaraju, Carswell, et al., 2009). By inhibiting the activity of these enzymes, the histones are more acetylated than usual, resulting in a decrease of the positive charge that normally allows the histones to bind to the negatively charged DNA. This causes the chromatin structure to open and affects the expression pattern of several genes, thereby possibly inducing cell cycle arrest and

apoptosis in colon cancer cells (Sivaprakasam et al., 2016). One possible pathway is that the chromatin modifications lead to the expression of a protein that stimulates the pathway by which mitochondria activate the protein caspase-3 and cause apoptosis in colorectal cancer cells (Medina et al., 1997).

In addition butyrate interacts with specific cell surface receptors (GPCRs or G-protein-coupled receptors). In the presence of a sufficient concentrations of butyrate or other SCFAs, these receptors are activated and transmit signals that could lead to the effects that are observed. With regard to colon cancer and butyrate, especially the GPR41, GPR43 and GPR109A receptors are of interest. The expression thereof is reduced significantly in colon cancer cell lines, but in presence of butyrate and upon restoring of their expression, cell cycle arrest and apoptosis are observed (Thangaraju, Cresci, et al., 2009). GPR41 responds to microbial SCFAs to mediate physiological responses of the host (Samuel et al., 2008). GPR43 is of interest because it may be a tumor suppressor based on the fact that butyrate and other SCFAs can signal through GPR43 to stimulate the expansion of regulatory T-cells in response to fiber, thereby regulating the inflammatory response.

Interestingly, previous research showed that butyrate-induced differentiation was associated with a lower cellular GSH level in HT-29 cells, especially during the onset of differentiation (Benard & Balasubramanian, 1997). Unfortunately, no glutathione assays were performed after the short-term exposure experiments with butyrate, but this could be of interest for future research, as this could confirm the hypothesis that differentiation occurred in this setup. On the other hand, GSH depletion was reported to decrease the rate of cell proliferation and to inhibit cancer growth (Terradez et al., 1993). For HT-29 cells exposed for 15 days to sub-toxic butyrate concentrations, the GSH level was indeed elevated in relation to untreated cells, which was manifested in a higher GSSG/GSH ratio, and was previously reported to stimulate mitosis (Shaw & Chou, 1986) and DNA synthesis (Suthanthiran et al., 1990), therefore contributing to cell proliferation. The higher GSH concentrations in the long-term experiment provides us with additional evidence that no differentiation has occurred as a result of low doses of butyrate, but that cell growth is stimulated.

The effects that were observed upon longer exposure with butyrate are opposite to the differentiation process, as cell proliferation is observed. Upon long-term exposure to sub-toxic concentrations, butyrate was probably used by the cells as an energy source, thereby stimulating cellular growth. The intracellular levels of butyrate were below the threshold required for growth inhibition and apoptosis, therefore not inducing the growth stop as seen in the short-term experiments (Csordas, 1996). An important remark for the long-term experiments is that butyrate treatment had much lower variances in the Seahorse assay than hemin and kynurenine, which implies that the cells easily adapt and shift to other pathways under influence of butyrate. Moreover, the Seahorse assay data showed a clear increase in basal and

total ACR for all tested concentrations, in parallel with an elevated ECAR; and the addition of oligomycin, which disrupts oxidative phosphorylation, substantially increased the OCR compared to the untreated cells. Both effects indicate that the energy production happens via a combination of mitochondrial and non-mitochondrial pathways. Our hypothesis is that the growth stimulation by butyrate is correlated to how glucose supplies energy to the cells. Butyrate possibly enters the Krebs cycle under the form of acetyl-CoA, thereby stimulating the energy supply for the cells, which is supported by a study of Bultman (2014). The mitochondrial oxidation of butyrate of isolated colonic epithelial cells was reported to account for 86% of the oxygen consumption, even in the presence of glucose (Roediger, 1982). The latter observation was probably a consequence of the suppression of glucose oxidation but not glycolysis by butyrate, through the production of acetyl-CoA. The extra acetyl-CoA decreases the activity of pyruvate dehydrogenase because of the changed ratio acetyl-CoA/free CoA. Moreover, a portion of butyrate is possibly converted to ketone bodies, which also act as a source of energy for the colonocytes (Roediger, 1982). Long-term exposure to butyrate probably promotes these pathways, and therefore also the mitochondrial respiration, which is manifested in the increased cell number that we perceived. This would also explain why we observed good mitochondrial respiration as well as acidification, which is then due to the glycolysis that continues because of the excess of glucose.

2.4 KYNURENINE

No short-term effect was visible when adding kynurenine as such to Caco-2 and HT-29 cells. Nevertheless, we observed an effect for the HCT 116 cells, which responded by higher mitochondrial activity, but not by cell growth. Overall, three days was thus not enough to see the activity of kynurenine. This was confirmed by the long-term experiments. Similar to the short-term exposure, the first days of kynurenine treatment had no significant effects on cell number or even induced some stress, whereas after this initial phase, recovery took place and the number increased rapidly compared to the untreated cells. Modifying the pathways thus took some time. This long initial lag phase can also be observed in the IncuCyte data, where the curves diverged only after some time.

Another remarkable observation is that the oxidative state of the cells was different after three days of treatment versus after 12 days of treatment. For HT-29 cells, no differences were seen. In contrast, HCT 116 cells experienced a higher oxidative stress than the untreated cells after the short-term experiments, as indicated by a significantly higher GSSG/GSH ratio, but a lower oxidative stress after the long-term exposure with kynurenine. The mechanisms that could account for the effects still remain underexplored, but we formulate some hypotheses.

Kynurenine is a primary katabolic product of the tryptophan pathway, which is an essential amino acid obtained by our diet. Kynurenine has been identified as a microbial conversion product present in the colon after consumption of processed meat (Rombouts et al., 2017). It has previously been associated with later stages of cancer progression. Munn and Mellor (2007) and Rombouts et al. (2017) reported a dual role for kynurenine in the context of cancer development. Cancer cells upregulate indoleamine 2,3-dioxygenase enzymes IDO1 and IDO and tryptophan dioxygenase (TDO), thereby consuming more tryptophan (Opitz et al., 2011; Prendergast, 2011; Rombouts et al., 2017) and creating more kynurenine. In addition, some intestinal microorganisms are known to produce IDO-enzymes (Yuasa & Ball, 2011; Yuasa et al., 2011). A first consequence of the accumulation of kynurenine metabolites is the effect on the immune-escape hallmark of cancer cells, which consists of the ability to escape from immune surveillance within the body (Thaker et al., 2013). Nearby immune cells create an important part of the cancer cell micro-environment, and a 'battle' between immune and cancer cells is continuously taking place. Kynurenine metabolites can promote host immunity escape by inhibiting the activation of T-cells and other immune cells. Moreover, kynurenine is an endogenous ligand for the aryl hydrocarbon receptor (AHR) of the cells. The binding of kynurenine to AHR results in the translocation of AHR to the nucleus, where it activates the transcription of target genes, eventually leading to a more invasive tumor. Moreover, the induced immune tolerance can spread from the tumor's micro-environment to the lymph nodes, which then also facilitates metastasis (Brandacher et al., 2006; Metz et al., 2007; Muller et al.; Munn & Mellor, 2007; Prendergast, 2011). Next to the immune escape, kynurenine metabolites are also believed to have a direct influence on the proliferation of cancer cells (Thaker et al., 2013). They directly activated nuclear β-catenin, which in turn stimulates targets of the Wnt signaling pathway in HT-29 and HCT 116 cells, thereby promoting the Wnt signaling. The Wnt pathway is a characteristic of malignancy and of CSCs (Vermeulen et al., 2010) and is known to promote the initiation of G1/S-phase transition within tumor cells, and thus cell proliferation (Thaker et al., 2013). The known effects of kynurenine are summarized in **Figure 23**. As a consequence of these pathways, IDO/TDO inhibitors gain interest as anticancer agents, especially when added concomitantly to other cancer treatments (Prendergast, 2011; Rombouts et al., 2017).
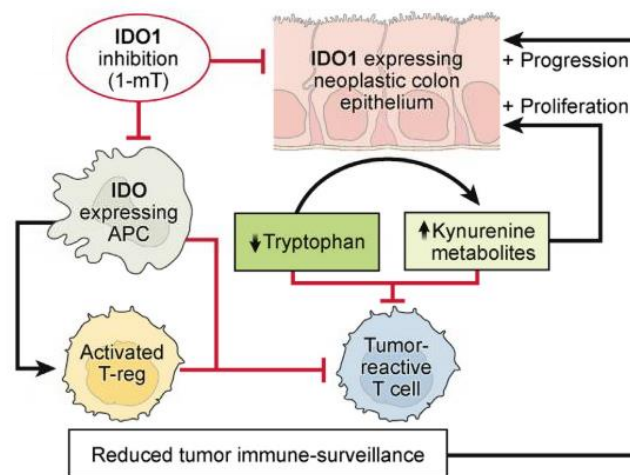
Figure 23: Schematic overview of the mechanisms by which the tryptophan pathway influences tumor cells (Thaker et al., 2013)

These pathways however do not completely explain the observations that kynurenine gave in the Seahorse assay, suggesting that this component might have more effects than is currently known. The Warburg effect was suggested by the dose-dependent yellow discoloration of the medium during the second long-term experiment, caused by lactate-formation. Given the effects on the cell numbers and on the Seahorse OCR and ECAR, there is most likely a relation with the energy production and growth mechanisms of the cells. We propose that the energy production of the cells under influence of kynurenine was based on other mechanisms than mitochondrial respiration and anaerobic glucose metabolism. As kynurenine is a product of the depletion of the amino acid tryptophan, there might be a link with the amino acid metabolism. Cancer cells are known to have an 'addiction' to another amino acid, being glutamine, as a consequence of some specific mutations. Glutamine provides the cells with building blocks but can also be used as a respiratory substrate, by conversion to α-ketoglutarate (Ganapathy et al., 2009; Roediger, 1982). Kynurenine could interfere with the glutamine pathway because of the structural similarities (**Figure 24**), thereby stimulating the cell growth.
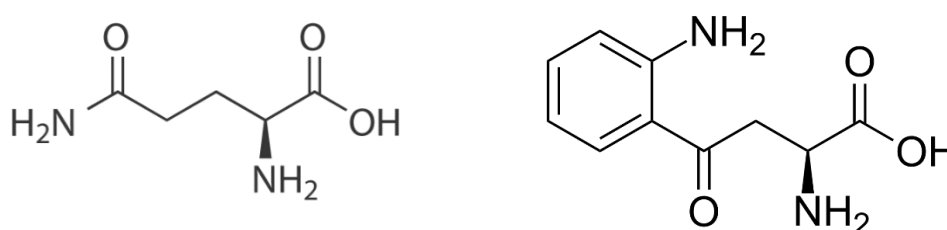

Figure 24: Molecular structure of glutamine (left) and kynurenine (right)

3 CELL LINE SPECIFIC EFFECTS

Cellular models have been of great value in the field of cancer research, by allowing to study the changes and mechanisms that drive the malignancy, and to determine potential biomarkers and mitigation strategies. Some treatments gave very different results for different cell lines, demonstrating that individually inherent characteristics of the cell lines influenced the way they coped with the presence of the components. The cell lines differ in stage of cancer, in appearance, in growth characteristics and harbor specific genetic characteristics (Ahmed et al., 2013). Moreover, they have different traits relating to oncogenic pathway activity, epithelial-mesenchymal transition (EMT) and stemness, migratory properties, proliferative activity, transporter expression profiles and chemosensitivity (Jon Christensen et al., 2012). For example, undifferentiated Caco-2 cells show the strongest Wnt signal of the three cell lines discussed here (J. Christensen et al., 2012), whereas HCT 116 cells owe their high invasiveness to the EMT, that is strongly present in this cell line (J. Christensen et al., 2012).

With regard to the genetic characteristics, variation is seen between the cell lines. The genes that are most commonly changed in CRC are APC, TP53, K-RAS, PIK3CA, B-RAF and PTEN (Ahmed et al., 2013). Each of the used cell lines can have the wild type gene or can harbor a mutation on the gene, resulting in different phenotypic properties of each cell line, as shown in **Table 6**.

Table 6: Overview of the mutations within each cell line of the most important genes involved in CRC development (Ahmed et al., 2013; GHR, 2017)

	HT-29	Caco-2	HCT 116	Effect
APC	Mutant	Mutant	Wild type	CRC initiation, B-catenin levels rise, Wnt signaling activated, stemness
K-RAS	Wild type	Wild type	Mutant	Warburg effect, uncontrolled cell growth and division
B-RAF	Mutant	Wild type	Wild type	Disruption of signaling involved in proliferation, differentiation, migration and apoptosis
PIK3CA	Mutant	Wild type	Mutant	Uncontrolled proliferation
PTEN	Wild type	Wild type	Wild type	Uncontrolled proliferation (later stages)
TP53	Mutant	Mutant	Wild type	Unregulated growth and division

These genetic differences, which are far more extended than the genes presented in the table, probably lie on the base of different effects that are observed during the experiments, as all other conditions were exactly the same for each cell line.

3.1 HEMIN

The response to hemin treatment was in the same line for all the cell lines, but the magnitude of the response was very different. Caco-2 cells were the most sensitive for the presence of hemin, and reacted by proliferating at sub-toxic concentration, both on short-term as long-term exposure, followed by the HT-29 cell line, where an increase in cell number was seen in the SRB assay on the short term, and a rising trend in cell number was observed during long-term exposure. The proliferation is not or barely detected for the HCT 116 cells, as hemin had no significant effect other than toxicity when added in high concentrations. We already suggested a link with the cancerous characteristics of the cell lines, which increase in the order of Caco-2, HT-29, HCT 116. The explanation is possibly also linked to the APC gene mutation, as it was reported that heme indeed promoted colonic cytotoxicity and hyperproliferation in normal cells, but not in premalignant cells, which are defined as APC+/+ and APC-/+ cells (Bastide et al., 2015). Caco-2 and HT-29 cells are mutant in the APC gene, whereas HCT 116 is wild type for APC (Ilyas et al., 1997), so HCT 116 is genetically similar for the APC gene to the group of cells that did not react to heme iron in the study of Bastide et al. (2015). Our results thus confirm that these premalignant cell lines respond significantly less to heme. This cell line specific reaction to heme treatment can be of interest for further investigation, as heme is thought to play a major role in the mechanisms through which the consumption of red meat increases the risk of colon cancer. HCT 116 is a cell line that represents a later stage of colon cancer, and as no direct effects are seen for these cells when treated with heme, the mechanisms involving heme are probably indirect, and rather target early stages of CRC.

3.2 BUTYRATE

Butyrate had a somewhat different effect on HCT 116 cells than on the other cell lines. Although butyrate treatments were not toxic for the cells in the short-term experiments, as was visually observed by checking the cells, the LDH value for HCT 116 cells increased significantly compared to the untreated cells, an effect that was not seen for the other two cell lines. A known trait of HCT 116 cells is that they have a higher mobility than the other two cells. This migratory capacity of HCT 116 cells over the HT-29 and Caco-2 cells is a consequence of the EMT, that is highly present in this cell line (J. Christensen et al., 2012), and probably also by the p53 gene, which is not mutated for HCT 116, in contrast to the other cell lines. Sablina et al. (2003) reported that loss of p53 function correlated with decreased cell migration, and a study of Hwang et al. (2011) concluded that wild-type p53 controls the cell motility and invasion, both explaining why HT-29 and Caco-2 cells are less mobile than HCT 116. We already hypothesized that the butyrate induced differentiation in the short-term experiments, thereby inducing a cell cycle arrest. Important is that differentiation goes hand in hand with a higher cellular motility (Mariadason et al., 2001; Rao et al., 1999). This added migratory capacity of the cells, combined with the fact that

HCT 116 cells already have a higher tendency to migrate because of their later cancer stage properties, the HCT 116 cells might have detached from the bottom of the plates, resulting in floating cells in the medium, which create an extra pool of LDH that is measured during the LDH assay. This cell line specific effect of butyrate should be confirmed in the future, for example by centrifuging the medium prior to the LDH measurement.

3.3 KYNURENINE

HCT 116 cells were the only cell line that responded to kynurenine on the short term, whereas all the cell lines reacted on long-term. One hypothesis is that HCT 116 cells exhibit a stronger glutamine-addiction, causing this response to kynurenine, as explained in the above mechanisms. This cancer hallmark is linked to mutations in the RAS, MYC and p53 genes (Amoêdo et al., 2013), and as shown in **Table 6**, HCT 116 differs from the other two cell lines on these genes. Also, HCT 116 is the most cancerous cell line of the three, which could also point to a higher glutamine-addiction. On the other hand, especially Caco-2 showed very high responses in cell number to long-term kynurenine exposure. This cell-line specific effect could be due to the link of kynurenine presence and the Wnt signaling pathway. As stated before, Caco-2 is known to have the strongest Wnt signaling of the three cell lines. As kynurenine affects this pathway, the large changes in Caco-2 cell numbers may be a consequence thereof.

4 ADHESION VERSUS GROWTH MECHANISMS

Two major mechanisms of the cells played a role during the first two long-term experiments, namely adhesion and growth. The cells were split during the experiment when confluency occurred, and were seeded again under certain conditions which included the test compound. Consequently, the cells had to attach again in presence of the component, which could also have an influence on the adhesion mechanisms. The response of the cells was only followed up by visual assessment and by cell number determination. To investigate if the observed effects on these parameters were the result of an impact on the cell adhesion or on cellular growth, the IncuCyte experiment and clonogenic assay were performed with very low seeding densities, which avoided the need for splitting during the ongoing assays. As the cells were never split during 12 days, and because the adhesion of the cells took place before the treatments with the components were added, extra effects that the components might have on the adhesion of the cells to the well surface were eliminated. Since the treatments gave similar results as the first two long-term experiments, especially for the IncuCyte assay, it is likely that the chosen components butyrate, hemin and kynurenine do not have an effect on the attachment process of the HT-29 cells. The observed effects can thus be attributed to an impact of the components on the growth mechanisms of the cells, which justifies the hypotheses made in the previous sections. In order to investigate the adhesion effects as such, a possible setup is to let cells attach in presence of the components, and to follow up the number of attached cells in the first hours after the seeding, where growth will still be limited.

Conclusions

A new *in vitro* approach that allows to expose cells in culture to pure component for a longer period of time was validated using colorectal cancer cell lines and red meat matrix components or microbial metabolites linked to the consumption of red meat. The results of the experiments matched the effects of the components as described in the literature, thereby supporting the success of the optimization of this innovative technique. Long-term exposure with sub-toxic concentrations of pure components induced different effects than when the component is added to the cells for only a short time. We could hypothesize that the cells need some time to establish a way of coping with it, which allows them to thrive even more than untreated cells, which manifests itself in higher cell numbers compared to untreated cells. This proves the high adaptability of cancer cells to environmental stimuli at very low concentrations. Especially Caco-2, but also HT-29 and HCT 116 cells on the longer term, were positively influenced by hemin with regard to growth and cell viability, which is of course negative for the patient. The hypothesis hereby is that the presence of heme had an impact on signaling pathways such as the Wnt signalling, resulting in a hyperproliferation and a more malignant phenotype of the cells, but also on the energy supply pathways (Warburg effect). Butyrate had two contrasting effects. On the short term, it most likely induced differentiation of the cells, thereby causing a cell cycle arrest. The mechanism involves the inhibition of HDAs, resulting in over-acetylation of histones, and deregulation of the expression of specific genes. Upon long-term exposure to much lower concentrations, the butyrate was not able to induce differentiation because the levels did not exceed the threshold value that is necessary. The growth was stimulated because colonocytes are able to metabolize butyrate as an energy source through infiltration of butyrate in the Krebs cycle in the form of acetyl-CoA or through the formation of ketone bodies. The kynurenine pathways remain unclear. It influences the mechanisms that create an immunosuppressive network, resulting in the protection of the tumor cells from the host immune attack, and stimulates tumor growth via the Wnt signaling pathway. Possibly, also an effect on the energy providing mechanisms is present. Cell line specific effects were explained by the genetic background of each cell line.

This research was unique in its long-term approach, but also the exploration of the effects of kynurenine exposure was never done before and might provide us with new insights in the mechanisms that link red meat consumption with CRC development. With these validated control components, the new approach could be used in the future to investigate other pure components and even digests. Anyhow, this offers potential to further explore the effect of red meat on our health.

Recommendations for future research

Using the same experimental setup but other assays, some controls could be done to confirm and further unravel the pathways behind the observed effects. For example, the Wnt pathway apparently plays a specific role in different mechanisms that stimulate the development of CRC, so a more profound investigation might provide new knowledge. Moreover, other markers could be used to confirm the differentiation by butyrate, such as the GSH level or dipeptidyl peptidase IV (DPP IV), a sensitive and specific marker for this differentiation process. Also some interactions are of interest, for example the IDO enzymes involved in kynurenine pathway are heme-catalyzed, suggesting that there are still effects that remain unclear.

The validated *in vitro* approach could then be applied in other setups. The components that were used here and of which the effects were matched with literature can be included as controls to verify the long-term tests as they are performed with for example *in vitro* or *in vivo* digests of red meat.

Moreover, the microbial metabolite related aspects of colorectal cancer development remain underexplored, especially in the context of red/processed meat consumption. By expanding the model further to for example cancer stem cells, or organoid cell culture, it becomes more complex and approaches the real tumor better and better, which would allow the investigation of the impact of the micro-environment to a greater extend.

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II

Supplementary data

ENDPOINT MEASUREMENTS LONG-TERM EXPOSURE TRIAL 2

The graphs of the endpoint assays after the second trial of a long-term exposure experiment are shown in **Figure 25.**
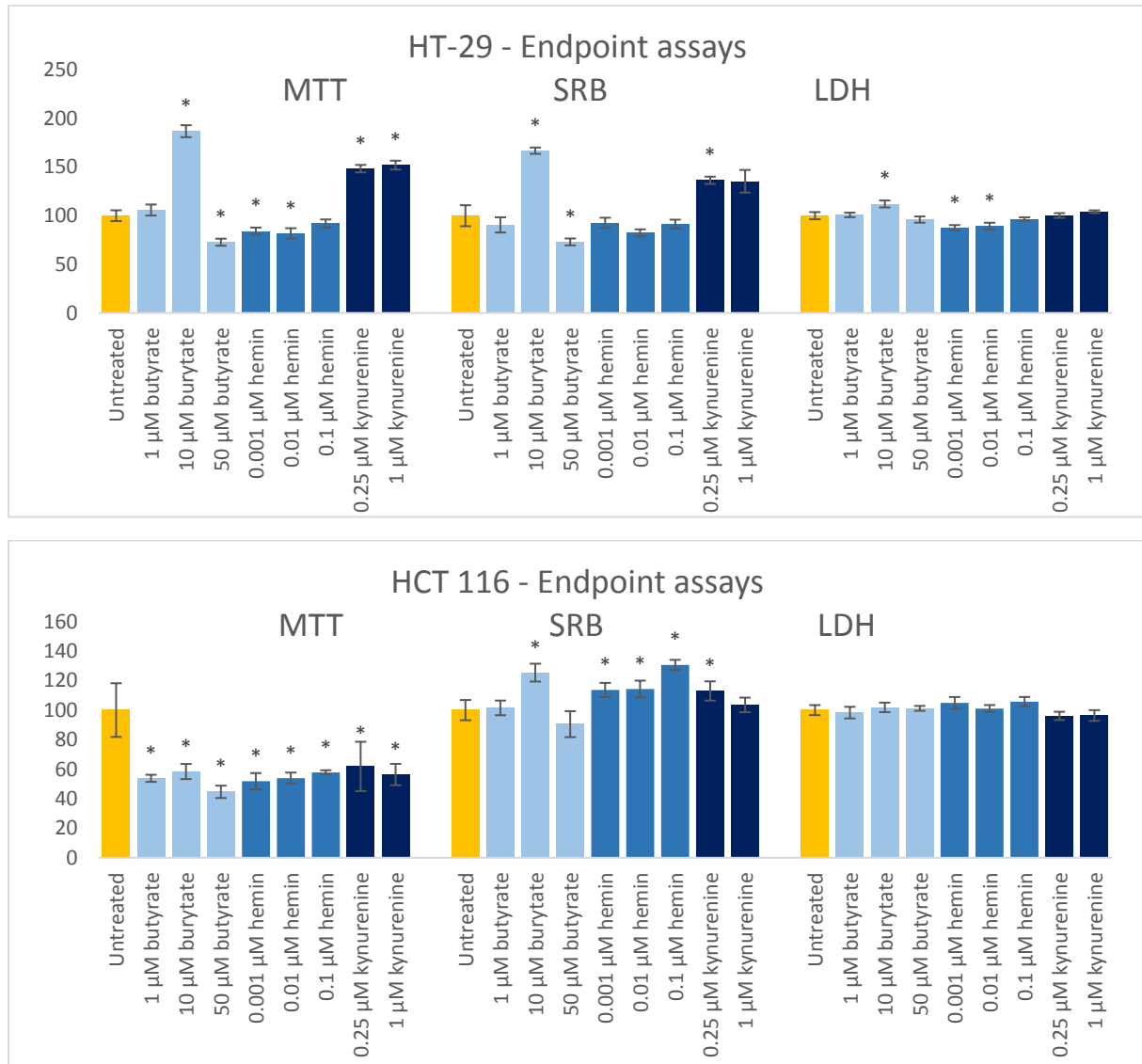

Figure 25: Results of the MTT, SRB and LDH assays on HT-29 and HCT 116 after the second long-term exposure experiment. An (*) indicates treatments that result in mean values that are significantly different from the value of the untreated cells. The MTT assay of HCT 116 cells should be interpreted with care as a problem occurred with the blanc cells. The color of these wells was completely different, probably due to a pH effect, causing a second absorbance peak..
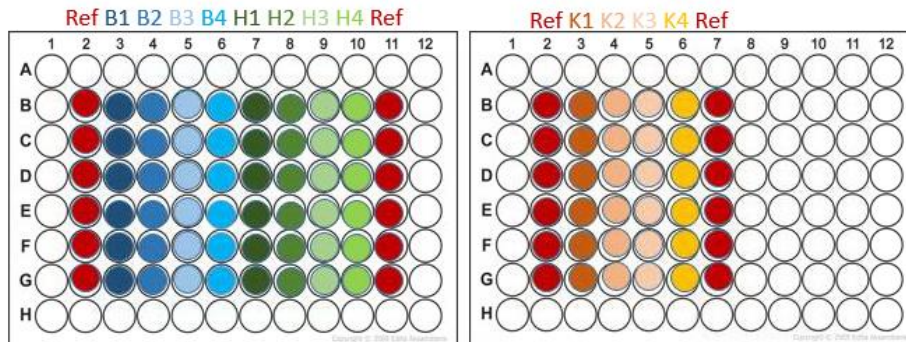
INCUCYTE®

SEEDING SCHEME

Figure 26: Schematic overview of the tested conditions in the IncuCyte® experiment. Ref: untreated cells, B1: 1 μM butyrate, B2: 10 μM butyrate, B3: 50 μM butyrate, B4: 100 μM butyrate, H1: 0.001 μM hemin, H2: 0.01 μM hemin, H3: 0.1 μM hemin, H4: 1 μM hemin, K1: 0.1 μM kynurenine, K2: 0.25 μM kynurenine, K3: 1 μM kynurenine, K4: 2.5 μM kynurenine.

VALIDATION STEP

A way to validate the IncuCyte assay is to check if the two columns with untreated cells generated similar growth curves. Therefore, **Figure 26** was made, where the reference conditions on one plate are compared to each other, for each of the two plates. The curves coincide quite well, only at the end of the experiment, there was some deviation, which is probably due to evaporation of the medium, which is often seen at the end of long-term experiments. Moreover, on plate 2, some contamination was observed in the second reference column after 12 days, so this could also explain the higher differences in plate 2 at the end.
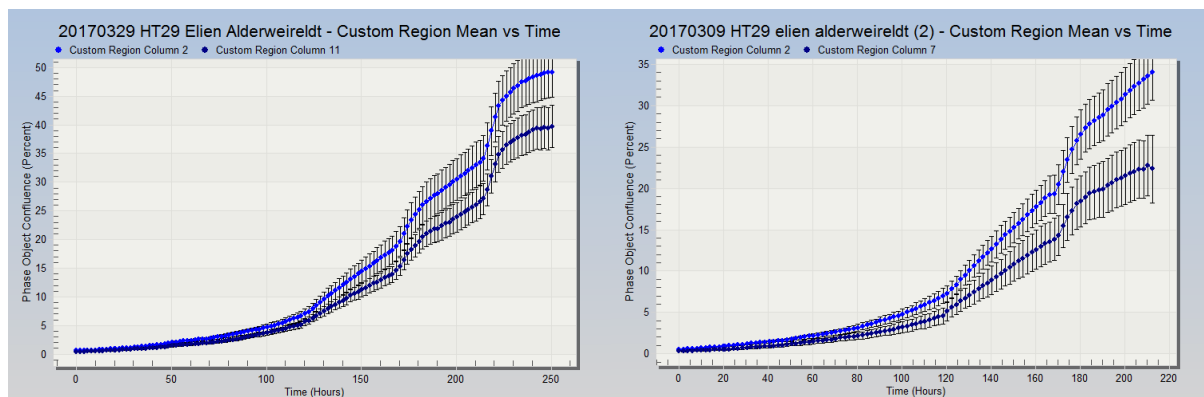

Figure 27: Confluency (%) in function of time of untreated HT-29 cells, comparison of two columns. Left: plate 1; right: plate 2

GROWTH PER WELL

The confluency (% of the surface of the well covered with cells) can be shown in function of time per well (**Figure 27**). This gives a nice overview of how the cells grew whilst undergoing the different treatments. Also here, in the second plate, the suspected contamination is visible as the cell numbers of the last two columns drop clearly at the end of the experiment. The data of the second plate is subsequently limited to the first 9 days, where no signs of contamination were present.
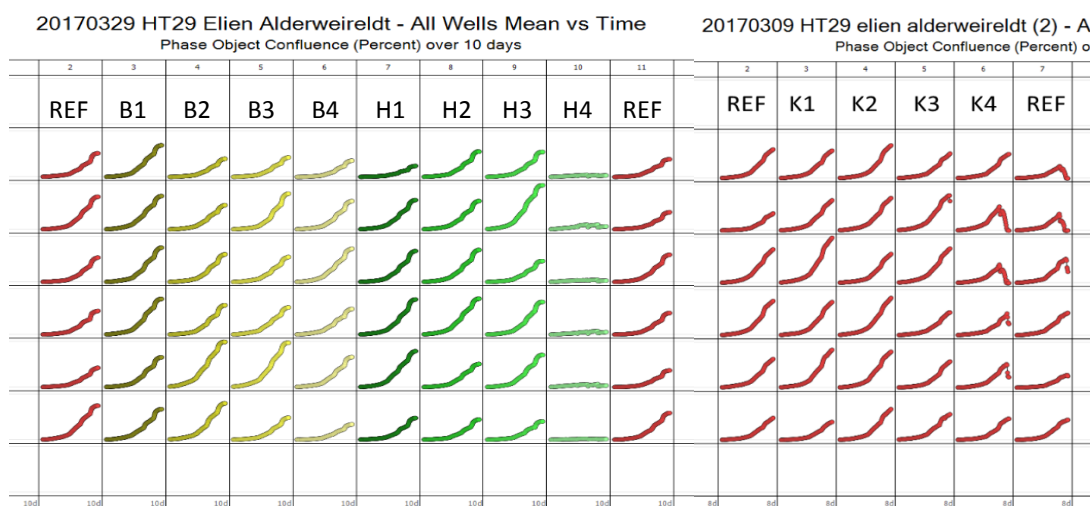

Figure 28: Confluency (%) of HT-29 cells in function of time per well. B1: 1 µM butyrate, B2: 10 µM butyrate, B2: 50 µM butyrate, B4: 100 µM butyrate, H1: 0.001 µM hemin, H2: 0.01 µM hemin, H3: 0.1 µM hemin, H4: 1 µM hemin, K1: 0.1 µM kynurenine, K2: 0.25 µM kynurenine, K3: 1 µM kynurenine, K4: 2.5 µM kynurenine.

DOUBLING TIME

The time that the cells need to go from a confluency of 10% to a confluency of 20%, as calculated by the IncuCyte® Software, can give an idea of the doubling time. The values are shown in **Table 8**.

The untreated HT-29 cells needed approximately 38 hours to grow from 10% confluency to 20% confluency. The butyrate treatments had a dose-response effect on this parameter: lower concentrations resulted in lower values, and thus faster growing cells; but as the concentration of butyrate increased, the effect was smaller and reversed. At a concentration of 50 µM, the doubling time was the same as the untreated cells, but a concentration of 100 µM caused a higher value, thereby indication that the cells grew slower at this concentration.

The hemin concentrations of 0.001 and 0.01 µM hemin induced a slight increase in the doubling parameter, which means that the cells grew a little bit slower than the untreated cells. Under influence of the highest hemin concentration, it took 64 hours for the cells to grow from 2% to 4% confluency, which proved that this treatment created a stressful environment and the cells barely grew.

The kynurenine treatments of 0.1 and 0.25 μM decreased the doubling time, whereas 1 μM kynurenine did not have any effect on this parameter. The highest concentrations again have the opposite effect, as the parameter increased. That implies that in low concentrations, kynurenine stimulates the growth of the cells, but when the concentration increases, the cell growth is decreased.

Table 7: Estimation of doubling time of the cells per condition

	Condition	Time to go from 10% to 20% confluency (hours)
	Untreated	38
B1	1 μM butyrate	30
B2	10 μM butyrate	34
B3	50 μM butyrate	38
B4	100 μM butyrate	40
H1	0.001 μM hemin	40
H2	0.01 μM hemin	40
H3	0.1 μM hemin	38
H4	1 μM hemin	64*
K1	0.1 μM kynurenine	34
K2	0.25 μM kynurenine	30
K3	1 μM kynurenine	38
K4	2.5 μM kynurenine	40

* time to go from 2% to 4% confluency