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BIO-INGENIEURSWETENSCHAPPEN

# INTRACELLULAR ACCUMULATION OF QUERCETIN AND ITS IMPACT ON CELLULAR STRESS: AN *IN VITRO* STUDY

Hanne Criel
Student number: 01407157

Promotor: Prof. dr. ir. John Van Camp
Tutors: dr. ir. Charlotte Grootaert
ir. Hanne Vissenaekens

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Prof. dr. ir. John Van Camp
Promotor

ir. Hanne Vissenaekens
Tutor

dr. ir. Charlotte Grootaert
Tutor

Hanne Criel
Author

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# ABSTRACT

Flavonoids belong to the polyphenol family and are, due to their wide distribution in the plant kingdom, a part of the daily human diet. The most ubiquitously present flavonoid, in a variety of fruits and vegetables, is quercetin. Despite the fact that flavonoids are not essential dietary factors, they are being investigated intensively because of their health-promoting properties associated with the prevention of various diseases, such as cardiovascular diseases and cancer.

Although many studies have shown the protective effect of flavonoids against cellular stress in cells, there is still an important lack of knowledge about which conditions influence the absorption of flavonoids and their conjugates, in order to exert their protective effect. In the present in vitro study, we investigate, using an intestinal cell line model, how cellular stress, induced by various stressors, can influence the absorption of the dietary flavonoid quercetin and how the accumulated quercetin may counteract cellular stress.

Our findings suggested that upon cellular stress, especially mitochondrial stress induced by oligomycin, FCCP and valinomycin, quercetin accumulation increased in proliferating intestinal cells. In addition, it was demonstrated that the accumulated quercetin improved the redox status and mitochondrial function by counteracting the (i) ROS production induced by oligomycin, FCCP and valinomycin, (ii) ATP depletion induced by FCCP, (iii) changes in mitochondrial membrane potential induced by all three stressors and (iv) FCCP induced shutdown of the mitochondrial respiration.

In conclusion, the results of this study may open perspectives for the approach of population stratification based on cellular stress for clinical trials. This may reveal a more consistent and significant impact of flavonoids on human health.

# SAMENVATTING

Flavonoïden behoren tot de familie van de polyfenolen en zijn, vanwege hun brede verspreiding in het plantenrijk, een onderdeel van ons dagelijkse dieet. Het meest frequent voorkomende flavonoïde, in een verscheidenheid aan groenten en fruit, is quercetine. Ondanks het feit dat polyfenolen geen essentiële voedingsfactoren zijn, worden ze intensief onderzocht vanwege hun gezondheidsbevorderende eigenschappen gerelateerd aan de preventie van verschillende ziekten, zoals hart- en vaatziekten en kanker.

Hoewel verscheidene studies hebben aangetoond dat flavonoïden de cel beschermen tegen cellulaire stress, is er zeer weinig geweten omtrent de omstandigheden die de absorptie van deze voedingscomponenten en hun metabolieten beïnvloeden. In de huidige *in vitro* studie werd onderzocht, met behulp van een intestinaal cellijnmodel, hoe cellulaire stress, geïnduceerd door inflammatoire en mitochondriale stressoren, de absorptie van de flavonoïde quercetine kan beïnvloeden en tot slot, hoe de geaccumuleerde quercetine cellulaire stress kan tegenwerken.

Onze bevindingen suggereerden dat na het induceren van cellulaire stress in de cel, voornamelijk mitochondriale stress, de accumulatie van quercetine toenam in de prolifererende darmcellen. Daarnaast werd aangetoond dat de geaccumuleerde quercetine de redoxstatus en mitochondriale functie verbeterde door (i) het verlagen van de ROS-productie geïnduceerd door oligomycine, FCCP en valinomycine, (ii) het tegenwerken van de ATP-depletie geïnduceerd door FCCP, (iii) het voorkomen van veranderingen in de mitochondriale membraanpotentiaal geïnduceerd door de drie stressoren en (iv) het tegenwerken van de FCCP geïnduceerde stopzetting van de mitochondriale respiratie.

Tot slot kunnen we concluderen dat de resultaten van deze studie perspectieven bieden voor de stratificatie van de populatie op basis van cellulaire stress voor het uitvoeren van klinische studies. Dit kan vermoedelijk een meer consistente en significante impact van flavonoiden op de menselijke gezondheid onthullen.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AP | activator protein |
| ATP | Adenosine triphosphate |
| BCRP | Breast Cancer Resistance Protein |
| CBG | Cytosolic-β-Glucosidase |
| COX | cyclooxygenase (COX) |
| DAMP | Damage associated molecular pattern |
| DCF | Dichloro-Fluorescein |
| DCFH-DA | Dichloro-Dihydro-Fluorescein Diacetate |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMSO | Dimethylsulfoxide |
| ECAR | Extracellular acidification rate |
| EDTA | Ethylenediaminetetraacetic acid |
| FBS | Fetal Bovine Serum |
| FCCP | Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone |
| GSH | Glutathione |
| GSSG | Glutathione disulfide |
| HO-1 | Heme oxygenase-1 |
| ICAM | intercellular adhesion molecule |
| IL | Interleukin (IL-1β, IL-6, IL-8) |
| iNOS | inducible nitric oxide synthase |
| IRF | interferon regulatory factor |
| JNK | c-Jun N-terminal kinase |
| LDH | Lactate dehydrogenase |
| LPH | Lactase Phloridzin Hydrolase |
| LPS | Lipopolysaccharide |
| MCP | monocyte chemoattractant protein |
| MRP | Multidrug Resistant Proteins |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5)-diphenyltetrazolium bromide |
| NEAA | Non-Essential Amino Acids |
| NF-κB | nuclear factor kappa B |
| NOD | Nucleotide oligomerization domain |
| Nrf2 | nuclear factor (erythroid-derived 2)-like2 |
| OAT | Organic Anion Transporters |
| OATP | Organic Anion Transporting Polypeptide |
| OCR | Oxygen consumption rate |
| OPT | O-phtalaldehyde |
| P/S | Pencillin/Streptomycin |
| PBS | Phosphate Buffered Saline |
| PGE2 | prostaglandin E2 |
| P-gp | P-Glycoprotein |
| PRR | Pattern recognition receptors |
| ROS | Reactive Oxygen Species |
| SGLT | Sodium-dependent Glucose Transporters |
| SLC | Solute Carrier transporters |
| SOD | superoxide dismutase |
| SULT | Sulfotransferase |
| TEER | Transepithelial electrical resistance |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| UGT | UDP-Glucronosyltransferase |
| VCAM | vascular cell adhesion molecule |

# INTRODUCTION

Flavonoids belong to the polyphenol family and are, due to their wide distribution in the plant kingdom, a part of the daily human diet. The most ubiquitously present flavonoid, in a variety of fruits and vegetables, is quercetin. Despite the fact that polyphenols are not essential dietary factors, they are being intensively investigated because of their health-promoting properties associated with the prevention of various diseases, such as cardiovascular diseases and cancer. Numerous studies have identified the active role of flavonoids against various types of stress and the mechanism of action has changed over time from the simple direct antioxidant hypothesis into a more complex pathway influencing molecular targets and cellular enzyme functions (Williamson, Kay, & Crozier, 2018).

Despite the fact that literature studies extensively demonstrated the bioactivity of flavonoids, their poor oral bio-availability hampers further development of these food components as therapeutic agents (B. Wu, Kulkarni, Basu, Zhang, & Hu, 2011). In addition, more recent epidemiological studies also showed that there are strong inter-individual differences in response to flavonoid consumption. A part of this variability can be explained by inter-individual differences in microbial composition and genetic background which both could influence flavonoid metabolism and absorption (Lampe & Chang, 2007). Moreover, it is also suggested that different host-related and microbial-related stimuli in the tissue environment, that can induce stress in the cells, may alter their response to the bioactive food component. Although many studies have shown the protective effect of flavonoids against cellular stress in cells, an important lack of knowledge is how these conditions influence the absorption of flavonoids and their conjugates, in order to exert their protective effect.

In the present in vitro study, an intestinal cell line model will be used to investigate how different types of cellular stress, induced by various stressors, can influence the absorption of the dietary flavonoid quercetin and how the accumulated quercetin may counteract cellular stress.

# 1 LITERATURE REVIEW

## 1.1 Introduction to flavonoids

### 1.1.1 General structure and dietary sources

Polyphenols are a class of secondary plant metabolites which are part of the human daily diet with fruit and fruit beverages (such as fruit juice, wine and tea) as main dietary sources. They are also found, to a lesser extent, in vegetables, dry legumes and cereals (Peterson & Dwyer, 1998; Winkel-Shirley, 2002). The most abundant class of polyphenols in the human diet are flavonoids (Gonzales et al., 2015). Flavonoids protect plants from different biotic and abiotic stress factors and function as detoxifying agents, signal molecules and antimicrobial components. Moreover, they play a functional role in frost hardiness, drought resistance, heat acclimatization and the freezing tolerance of plants (Agati et al., 2012; Panche et al. 2016; Winkel-Shirley, 2002).

It has been reported that the consumption of fruit and vegetables may reduce the risk of developing cancers (Steinmetz & Potter, 1996) and stroke (Ness & Powles, 1997). In addition, studies by Yang et al. (1993) and Tijburg et al. (1997) showed that the consumption of tea may protect against cancers and coronary heart diseases, respectively. Even the consumption of wine is linked to the prevention of coronary heart disease (Criqui & Ringel, 1994). Moreover, epidemiological studies suggest indeed association between the consumption of flavonoid-rich foods or beverages and the prevention of a large number of diseases, including cancer, cardiovascular disease, and neurodegenerative disorders (Bobe et al., 2008; Hertog et al., 1993; Kris-Etherton & Keen, 2002; Mennen et al., 2004; Spencer, 2010) although it should be noted that some studies report inconclusive results with some illustrating the high inter-individual response to flavonoid consumption (Kay et al., 2012). Nevertheless, there is nowadays an immense interest in research on flavonoids.

The broad spectrum of health-promoting effects is associated with the antioxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic properties of flavonoids coupled with their capacity to modulate key cellular enzyme functions (Kumar et al., 2016). In contrast, some studies strongly suggest the pro-oxidative and pro-inflammatory properties of excess flavonoids *in vitro* hence, promoting oxidative damage (Halliwell, 2008). According to Procházková et al. (2011), pro-oxidant or antioxidant properties of a particular flavonoid depends most of all on its concentration in the cell. However, it is important to notice that their pro-oxidant effects can also be beneficial and not only toxic, since a mild degree of oxidative stress raises the levels of antioxidant defenses, leading to overall cytoprotection (Halliwell, 2008).

The bioavailability, metabolism, and biological activity of flavonoids depends upon the configuration, total number of hydroxyl groups, and substitution of functional groups on their basic phenolic structure (Fout! Verwijzingsbron niet gevonden.) (Panche et al., 2016).

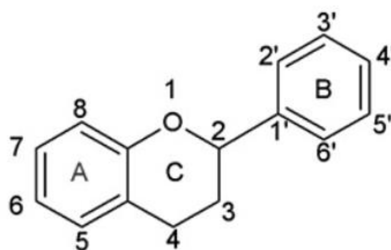


*Figure 1: Basic structure flavonoid (Panche et al., 2016)*

Based on (i) the position of the B-ring on the C-ring, (ii) the degree of unsaturation of the C-ring and (iii) the oxidation state of the C-ring, flavonoids can be subdivided into different groups. The most common subgroups are: flavones, flavonols, flavanones, isoflavonoids, anthocyanins and chalcones (Panche et al., 2016). Fout! Verwijzingsbron niet gevonden. gives a summary of these flavonoid classes, their basic structure and some examples.

*Table 1: Summary of the most important flavonoid classes and some examples (Panche et al., 2016)*

| Flavonoids | | | | | | |
|---|---|---|---|---|---|---|
| **Classes** | Flavones | Flavonols | Flavanones | Iso-flavonoids | Anthocyanins | Chalcones |
| **Basic structure** | | | | | | |
| **Examples** | Apigenin<br>Tangeretin<br>Baicalein | Quercetin<br>Myricetin<br>Rutin<br>Morin<br>Kaempferol | Hesperitin<br>Naringin<br>Naringenin<br>Eriodictyol<br>Hesperidin | Genistin<br>Genistein<br>Daidzein<br>Glycitein<br>Daidzin | Cyanidin<br>Malvidin<br>Delphinidin<br>Peonidin | Phloretin<br>Arbutin<br>Phlioridzin |

The structural diversity of dietary flavonoids is further complicated by (i) hydroxylation, (ii) glycosylation and (ii) acylation with phenolic acids, of the phenolic rings (Scalbert & Williamson, 2000). The structural diversity of flavonoids makes the estimation of their content in food especially difficult (Manach et al., 2004). The most abundant flavonoids in the human diet are flavonols, anthocyanins and their oxidation products (Scalbert & Williamson, 2000). In addition, one of the most studied flavonoids, omnipresent in the human diet, in a variety of fruits and vegetables such as onions, grapes, and cauliflower, is quercetin (de Oliveira et al., 2016). Therefore, this flavonol will be used as model flavonoid in the experimental part of the master dissertation.

## 1.1.2 Absorption, metabolism and bioavailability of dietary flavonoids

### 1.1.2.1 Cellular uptake and transport

The cellular absorption of flavonoid (metabolites) depends on different parameters such as cell type and the chemical structure of the flavonoid, etc. (Panche et al., 2016). Additionally, the gastrointestinal absorption and metabolism of flavonoids may also be influenced by the food matrix. For example, the absorption of quercetin from onions is fourfold greater than absorption from apple or tea (Le Marchand,

2002). A major difference between these sources is the type of glycoside: onions contain only quercetin glucosides whereas tea and apple also contain other glycosides. On the other hand, the influence of the food matrix on the bioavailability of flavonoids may also occur because of (i) direct interactions between the flavonoid and the food components, (ii) indirect effects of the diet on the gut physiology or (ii) induction or inhibition of enzymes and carriers, involved in flavonoid absorption and metabolism, by the presence of certain micronutrients or xenobiotics (Hollman et al., 1997; Manach et al., 2004).

In general, the main flavonoid absorption pathway is passive diffusion through the cell membrane. The transport rate depends in this case on the hydrophobicity of the molecule (Panche et al., 2016; Scalbert & Williamson, 2000). For example, flavonoid aglycones are, in general, easily absorbed into the cell since their lipophilicity facilitates easy passage across the phospholipid bilayer (Kroon et al., 2004). Besides passive diffusion, selective transport with membrane-associated transporters plays also a crucial role in cellular flavonoid uptake (Gonzales et al., 2015). A first important type of membrane transporters are the solute carriers (SLCs), including organic anion transporting polypepetide (OATP), organic anion transporters (OAT) and monocarboxylic acid transporters (MCT) (Panche et al., 2016). The selective uptake of flavonoids by a specific transporter depends on the recognition of certain flavonoid moieties by that transporter. For example, OAT4 and OATP4C1 are highly present in the human hepatocellular carcinoma cell line (HepG2) (Wong et al., 2012). These transporters prefer sulfated over glucuronide conjugates which explains the high uptake of quercetin-3'-O-sulfate and the lack in quercetin glucuronide uptake, by this cell type (Gonzales et al., 2015). Other membrane proteins, that are suggested as transporters that mediate the uptake of various flavonoids, are sodium glucose linked transporters (SGLTs) and bilitranslocase (Gonzales et al., 2015). On the other hand, the ATP-binding cassette (ABC) transporters, such as multidrug resistant proteins (MRP), P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) are responsible for the efflux of flavonoids out the cell (Crozier et al., 2010). This way, the ABC transporters affect the availability and distribution of flavonoids in the tissue and in turn, their beneficial effects (Alvarez et al., 2010). Moreover, it has also been observed that flavonoids are not only substrates of the ABC transporter, but they can also induce (Alvarez et al., 2010) or inhibit (Brand et al., 2006) ABC transporter activity.

It is established that the composition of membrane transport proteins including influx and efflux transporters is different between different cell types. In addition, studies also show that the composition of the transporters changes during inflammation or disease states. For example, Holla et al. (2008) were not able to detect OATP2A1 in several colorectal cancer cell lines, including HCT 116 cells and Lee et al. (2008) reported that the normally liver-exclusive OATP1B3 is also expressed in gastric, colon and pancreatic cancers, including HCT 116 cells, with the exception of Caco-2 cells. Additionally, inflammatory bowel disease is associated with elevated OATP2B1 and OATP4A1 levels in ileum and colon (Wojtal et al., 2009).

Moreover, the action of each of these transporters may depend on certain factors such as extracellular pH, membrane potential, amount of glucose, amount of energy (ATP) and the presence of certain

inhibitors or inducers. For example, studies have shown that OATP transport activity is increased at acidic pH suggesting that quercetin may be acting as a high-affinity substrate of OATP in order to enter the cell at low pH values (Leuthold et al., 2009). Moreover, a study by Glaeser et al. (2014) that studied the influence of extracellular pH on the OATP2B1-mediated quercetin uptake, showed that the uptake of quercetin (0.1 µM) into the HEK293-OATP2B1 and the HEK293 control cells, significantly decreased with increasing pH from pH 5.5 to pH 7.4 and from pH 7.4 to pH 8.5. Changing the extracellular pH can either affect the charge of the substrate or the substrate binding pocket of the transporter. It has been proposed that a decrease in extracellular pH increases the substrate affinity (lower $K_m$ values) because of the protonation of a conserved histidine residue at the extracellular end of transmembrane domain 3 (Leuthold et al., 2009). This was illustrated by Leuthold et al. (2009) that in Caco-2 cells, OATP transporters are responsible for transport of quercetin at low pH, whereas passive diffusion is responsible for transport at higher pH. Additionally, OATP substrate transport generally leads to stimulation of bicarbonate or glutathione efflux supporting the concept that OATP transporters act as anion exchangers. In addition, flavonoids have also been found to modulate SLC activity (Gonzales et al., 2015). For example, Glaeser et al. (2014) reported that apigenin and quercetin inhibit transport mediated by certain SLCs in kidney cells.

#### 1.1.2.2 Intracellular accumulation

Studies concerning intracellular flavonoid accumulation are still rare. However, Mukai et al. (2011) used confocal laser scanning fluorescence microscopy to demonstrate the subcellular localization of flavonol aglycones in different intact cell lines. The results showed that quercetin accumulates in the nucleus of human umbilical vein endothelial cells (HUVEC) and is associated with the cellular membrane of human colon carcinoma (Caco)-2 cells. The latter observation was confirmed by Gonzales, Smagghe, et al. (2016) using confocal microscopy and flow cytometry. It was suggested that flavonoids are incorporated in the cellular membrane with the polar head group of the phospholipid. Additionally, Notas et al. (2012) detected that quercetin was predominantly present in the nucleus in the HepG2 cells and Fiorani et al. (2010) reported that quercetin accumulates in the mitochondria of Jurkat human T lymphoblast cells.

#### 1.1.2.3 Metabolism

The apical side of the intestinal cells comes mostly into contact with flavonoid glycosides (fig. 2) since these are the most frequent forms of flavonoids present in food (Perez-Vizcaino et al., 2012). In vitro studies show that the cellular uptake of glycosides in intestinal Caco-2 cells is at least five times lower than their aglycones (fig. 2) (Perez-Vizcaino et al., 2012). A first explanation for this observation is the slow passive diffusion of glycosides because of their poor lipid solubility in contrast to the high lipophilicity of aglycones. A second reason is their poor uptake by most membrane transporter proteins, with the exception of SGLT-1 and finally, their fast efflux by most ABC transporters. In order to obtain a better cellular uptake, glucosides are hydrolyzed into their aglycone form by β-glucosidases such as lactase phloridzin hydrolase (LPH) in brush-border of the small intestine epithelial cells or by cytosolic β-glucosidase (CBG) within the epithelial cells after absorption of the flavonoid glucosides by (SGLT-1) (Kroon et al., 2004). It has been observed that LPH has a substrate specificity for flavonoid-O-β-

glucosides which could explain the fast absorption of quercetin glucosides in onions as described in section 1.1.2.1 (Kroon et al. 2004). Both LPH and CBG are expressed by the Caco-2 cell line and ex vivo human small intestinal samples. However, β-glucosidase activity in Caco-2 cells is much lower compared with actual intestinal tissue samples. Glycosides which are not deglycosylated or absorbed in the small intestine are transported towards the colon where they can be hydrolyzed by the colon microbiota (Cook & Samman, 1996; Kroon et al., 2004).

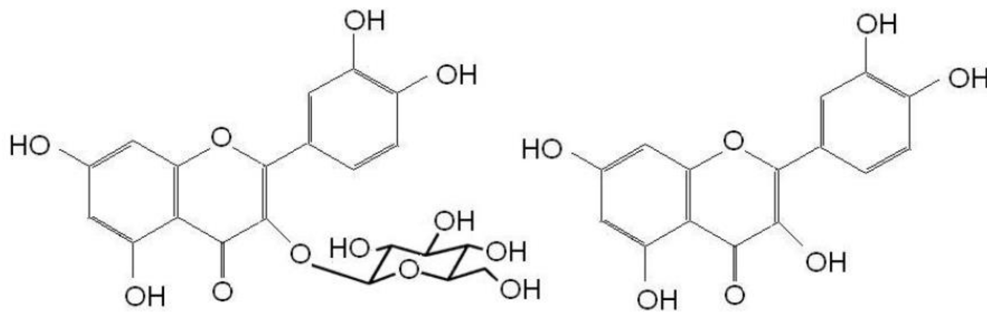


*Figure 2: Structure of a flavonoid glycoside (left) and flavonoid aglycone (right) (Peterson & Dwyer, 1998)*

In contrast to *in vitro* studies, *in vivo* studies suggest that increasing the aqueous solubility of flavonoids (more glycosides instead of the aglycone form) improves their bioavailability. For this reason, Gonzales, Van Camp, et al. (2016) suggested that the mucus layer on the epithelial cells participates in the absorption of flavonoids in the intestinal cells since the hydrophilic nature of this layer can inhibit the passage of hydrophobic polyphenols (aglycones) and only allows the passage of hydrophilic compounds (flavonoid glycosides). In case of the latter, hydrolysis occurs after the compound has penetrated through the mucus by β-glucosidases located in the brush-border. On the other hand, hydrophobic flavonoid aglycones are unable to penetrate through the mucus layer and are thus metabolized in the large intestine. However, dietary fat and bile can serve as carriers of aglycones through the mucus layer by formation of micelles (Gonzales, Van Camp, et al., 2016).

After absorption, by passive diffusion or through membrane transporters, the flavonoid aglycones are further metabolized in the intestinal cells by metabolizing enzymes (Gonzales et al., 2015). The most frequent metabolizing pathways in intestinal cells are glucuronidation, mediated by UDP-glucuronosyl-transferases (UGTs) and methylation, mediated by methyltransferases (COMTs) (Kumar & Pandey, 2013; Perez-Vizcaino et al., 2012). In addition, sulfation of the flavonoids is possible since it is known that Caco-2 cells, TC7 cells and human intestinal mucosa are rich sources of sulfotransferase (SULT) (Kroon et al. 2014). For example, a study by Gonzales, Van Camp, et al. (2016), showed that undifferentiated Caco-2 cells convert quercetin aglycones into several conjugates in the culture medium by cellular metabolism including glucuronides and sulfates. However, the majority of the metabolites in the medium were methylated derivatives since the catechol structure in quercetin makes it very prone to O-methylation by COMT. Moreover, quercetin aglycone and methylated forms were found intracellular. **Figure 3** shows a simplified overview of the metabolism of flavonoids in the gut and in enterocytes.

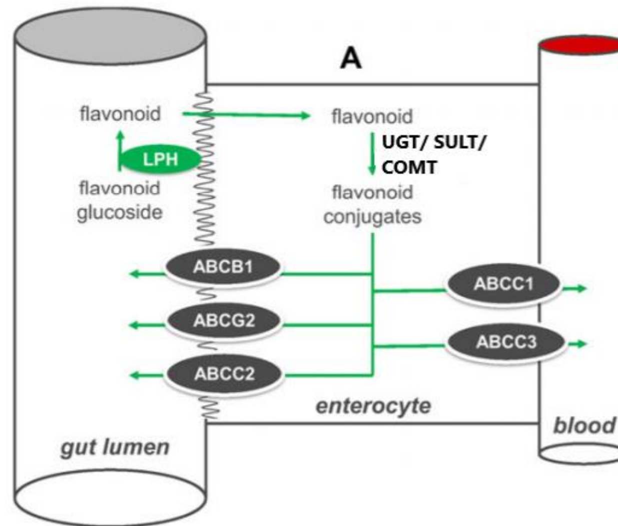


*Figure 3: Simplified overview of the absorption and metabolism pathway of flavonoids in the intestinal enterocyte. Flavonoid glucoside is hydrolyzed into the aglycone form by LPH. After absorption, the aglycone is metabolized by metabolizing enzymes after which flavonoid conjugates are formed. Those conjugates are transported out of the cell by a range of ABC transporters, back into the gut or into the bloodstream (Williamson et al., 2018)*

Moreover, flavonoid conjugates formed in the intestinal cells are then transported to the liver through the portal bloodstream in which they are further methylated, glucuronidated or sulphated. Some flavonoid metabolites produced in the liver can be recycled back into the small intestine through bile excretion (Crozier et al., 2010). Because of the high metabolism of the flavonoids in the gut and the liver, mostly conjugated flavonoids, such as glucuronides and sulfates, with or without methylation, are found in the plasma and the aglycone form is hardly present (Kroon et al., 2004; Scalbert & Williamson, 2000). In addition, endothelial cells come mostly into contact with those conjugated forms and not with the reactive aglycone form. Since most flavonoid conjugates have a hydrophilic character, they can only cross the lipid rich cell membranes of the endothelial cells with the help of sodium-glucose-associated carrier protein active transporters (SGLT), which are expressed in endothelial cells (Gonzales et al., 2015). However, cellular uptake of flavonoid conjugates through this pathway is limited when ATP production is disrupted. Since the lipophilic aglycone form is much more easily absorbed then the hydrophilic conjugated forms, it is interesting to mention that the conjugated forms can be transformed back into the aglycone form by the lysosomal enzyme β-glucuronidase during deglucuronidation (Gonzales et al., 2015). Shimoi and Nakayama (2005) confirmed that four types of human cells (neutrophils, HUVEC, IMR-90, and Caco-2) possess β-glucuronidase activity. Among these, Caco-2 cells showed the highest level of β-glucuronidase activity. The flavonoids that are absorbed in the endothelial cell (in aglycone or conjugated form) can be further metabolized. Gonzales et al. (2015) suggested that sulfation, methylation and glucuronidation can all occur in endothelial cells.

In general, the aglycone form is seen as the most bioactive form of flavonoids. For example, on average, is the antioxidant capacity of quercetin conjugates about half of the antioxidant capacity of quercetin aglycone (Kroon et al., 2004). Interestingly, more recent studies report increased or differential activity

of some flavonoid metabolites on multiple enzyme systems and signaling pathways (Kroon et al., 2004; Williamson, Kay, & Crozier, 2018).
As a result of this extensive metabolism, molecular forms of flavonoids reaching the blood flow and tissues are in general completely different from the molecular forms present in the diet (Kroon et al., 2004). In addition, the final concentrations achieved in the plasma are in many cases below the micromolar range. According to Scalbert and Williamson (2000) the plasma concentration rarely exceeds 1 µM after consumption of 10-100 mg flavonoid. This low bioavailability is probably a result of the (i) specificity of the transporters and enzymes in the gastrointestinal tract, (ii) efflux of flavonoids back into the intestinal lumen via ABC transporters and bile excretion and (iii) extensive metabolism of the flavonoids in the intestinal wall, the liver and the peripheral tissue, as described above (Manach et al., 2004; Perez-Vizcaino et al., 2012).

### 1.1.3 Flavonoid paradox

The health promoting effects associated with flavonoids are discussed in section 1.1.1 of this literature review, while in section 1.1.2, it was concluded that the bioavailability of flavonoids is low. This contradiction, the high bioactivity of flavonoids despite their low bioavailability, is referred to as the "flavonoid paradox" (Perez-Vizcaino et al., 2012).

The flavonoid paradox may be partially explained by the hypothesis that flavonoid uptake, metabolism and accumulation changes upon cellular stress. For example, it has been described before that β-glucuronidase activity in human neutrophils increases upon cellular stress, induced by bacterial endotoxin LPS, resulting in more conversion of the flavonoid glucuronide into the active aglycone (Shimoi et al., 2001). Moreover, Ishisaka et al. (2013) reported an increase in β-glucuronidase activity by RAW264 cells upon mitochondrial dysfunction induced by antimycin-A, also resulting in the stimulation of the conversion of quercetin glucuronide into quercetin aglycone. Furthermore, Gonzales, Smagghe, et al. (2016) described that upon mitochondrial dysfunction, induced by the ionophore valinomycin, the intracellular accumulation of O-methylated quercetin was higher in Caco-2 cells compared to cells treated with quercetin alone. Interestingly, also the localization of quercetin inside the cells changed. Quercetin normally accumulates at the cell membrane (Mukai et al., 2011), whereas treatment with valinomycin showed that quercetin also penetrated into the cytoplasm (Gonzales, Smagghe, et al., 2016). These findings demonstrated a change in localization, cellular uptake and metabolism of quercetin induced by valinomycin, which suggests a potential cellular stress response mechanism in Caco-2 cells.

Based on the results of these studies, it is interesting to further investigate the influence of cellular stress on the bioactivity and bioavailability of flavonoids as a possible explanation of the flavonoid paradox.

## 1.2 Introduction to cellular stress

Cellular stress can be induced by different stimuli or stressors such as tissue damage, toxic components, pathogens, heat, UV radiation, oxidative stress etc. This may result in the disruption of the homeostasis and subsequently the initiation of several cellular stress responses which will counteract stress-induced damage and remove terminally damaged cells by programmed cell death (apoptosis) (Fulda et al., 2010; Kültz, 2005). Some aspects of this cellular stress response are stressor specific, others are activated as a result of any type of macromolecular damage such as damage to DNA, proteins and lipids (Fulda et al., 2010; Kültz, 2005).

The cellular stress response is essential to the survival of cells confronted with environmental stimuli. However, long term cellular stress and subsequently long term cellular stress responses enhance the susceptibility to diseases, such as neurodegenerative diseases, diabetes, cardiovascular diseases and cancer (Fulda et al., 2010; Milisav, 2011). In the following sections, we first focus on a very important aspect of the cellular stress response: inflammation (section 1.2.1). In section 1.2.2, we discuss cellular stress induced by mitochondrial dysfunction and its link with the inflammatory response. Furthermore, stimuli to induce the inflammatory pathway and mitochondrial dysfunction in *in vitro* models are discussed.

### 1.2.1 Inflammation

Inflammation is a natural and biological process that contributes to physiological and pathological processes (Hansson, 2005; Libby, 2012; Ryan & Majno, 1977; Wyss-Coray & Rogers, 2012). There are two stages of inflammation, acute and chronic inflammation (Kültz, 2005). Acute inflammation is an initial stage of inflammation, associated with wound healing and fighting infections, which only persists for a short period of time. If the inflammation lasts for a longer period of time, the second, chronic, stage of inflammation sets in which increases the risk of various chronic illnesses (Coussens & Werb, 2002; Hansson, 2005; Wellen & Hotamisligil, 2005). Chronic inflammation is mainly linked with inflammatory bowel diseases (IBDs) (Van De Walle et al., 2010) and atherosclerosis, which is the primary cause of heart disease and stroke (Lusis, 2000). Furthermore, chronic inflammation is also linked to diseases such as Alzheimer's disease (Wyss-Coray & Rogers, 2012), diabetes (Wellen & Hotamisligil, 2005) and cancer (Coussens & Werb, 2002).

#### 1.2.1.1 The inflammatory response

The inflammatory response is activated in response to various stress stimuli including tissue injury, microbial pathogen infection and oxidative stress (Takeuchi & Akira, 2010). The presence of stress stimuli is detected by germline-encoded pattern recognition receptors (PRRs) (Takeuchi & Akira, 2010). These receptors recognize pathogen-associated molecular patterns (PAMPs), which are structures conserved among microbial species, such as lipopolysaccharide (LPS). On the other hand, PRRs are also able to recognize endogenous molecules released by damaged cells, called damage associated molecular patterns (DAMPs), including mitochondrial content, DNA fragments, ATP, IL-1α and uric acid (Kaczmarek et al., 2013; Kumagai & Akira, 2010; Kyriakis & Avruch, 2012; Takeuchi & Akira, 2010).

PRRs can be subdivided into four different classes including transmembrane proteins such as the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as cytoplasmic proteins such as the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs) (Kumagai & Akira, 2010). These PRRs are expressed not only in immune cells such as macrophages and dendritic cells but also in various non-immune cells such as epithelial cells (Cario et al., 2000) and endothelial cells (Dauphinee & Karsan, 2006; Faure et al., 2000).

The activation of the PRR signaling pathways causes the upregulation of a set of transcription factors such as nuclear factor kappa B (NF-κB), interferon regulatory factors (IRFs) and activator protein-1 (AP-1) (Takeuchi & Akira, 2010). Upregulation of these transcription factors will subsequently lead to the upregulation of the expression of gene products including pro-inflammatory cytokines, anti-inflammatory cytokines, interferons, chemokines and antimicrobial proteins. All of those PRR-induced proteins are involved in the positive and negative regulation of the inflammatory response (Takeuchi & Akira, 2010). Either way, pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6 are secreted by monocytes, such as THP-1 (X. Zhang et al., 2014) and RAW 264.7 cells (Nishitani et al., 2013; G. Yang et al., 2013), but also by endothelial HUVEC cells (Y. Zhou et al., 2018). These cytokines orchestrate the inflammatory response by modifying the vascular endothelial permeability (Jia et al., 2015; T. H. Kim et al., 2010; W. Lee et al., 2014), increasing the production of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (Cho et al., 2016; T. H. Kim et al., 2010; Shalini et al., 2016) by endothelial cells, and enhancing the production of chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) by HT-29 cells (Amasheh et al., 2012) and HUVEC cells (Shalini et al., 2016). This way, the recruitment of leukocytes to the affected tissue is increased.

An increase in pro-inflammatory cytokines, such as TNF-α, stimulates the production of reactive oxygen species (ROS) (Federico et al., 2007). In turn, oxidative stress, i.e. when the level of reactive oxygen species (ROS) exceeds the natural antioxidant defense of the cells, may induce the inflammatory pathway. Moreover, reactive oxygen species can directly stimulate inflammation by activating transcription factors, such as NF-κB or indirectly since oxidative stress can also damage macromolecules such as DNA, proteins and lipids, leading to DAMP-induced inflammation (Reuter et al., 2010).

Furthermore, activation of the transcription factors NF-κB and AP-1 by PRR sensing is highly associated with the induction of inflammatory enzymes, including inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 (Park & Song, 2013). Overproduction of NO by iNOS and prostaglandin-E2 (PGE2) by COX-2, are common markers of chronic inflammation (Park & Song, 2013).

#### 1.2.1.2 *In vitro* simulation of inflammation

##### 1.2.1.2.1 TNF-α

Using cell line models and pro-inflammatory stimuli, inflammation and the inflammatory response can be studied *in vitro*. For example, the pro-inflammatory cytokine TNF-α, which is part of the tumor necrosis factor (TNF) super family, can be applied to induce inflammatory stress *in vitro*. TNF-α is primarily produced by activated leukocytes, but also by other cell types including lymphoid cells, mast cells, endothelial cells (such as EA.hy926 cells and HUVEC cells), cardiac myocytes, fibroblasts, neurons and intestinal cells (such as Caco-2 cells) (Jia et al., 2015).

According to Blaser et al. (2016), starts the TNF induced inflammation pathway with the binding of TNF, which can be soluble (sTNF) or membrane-bound (mTNF), to two possible TNF receptors: TNFR1 and TNFR2. TNFR1 is expressed in almost all cell types and can be activated by both sTNF and mTNF, whereas TNFR2 is restricted to immune and endothelial cells. Either way, the stimulation of the TNF receptors by TNF can lead to the activation of nuclear factor κB (NF-κB). NF-kB is present in the cytoplasm in an inactive complex bound to inhibitor IkB proteins and can be activated by the phosphorylation-induced degradation of IkB (Karin, 1999). The binding of TNF to the receptor on the cell stimulates the activity of the IkB kinase (IKK) complex which catalyzes this event. In addition, besides the phosphorylation and subsequent degradation of inhibitory molecules, functional domains of the NF-kB proteins themselves, such as p65, need to be phosphorylated by protein kinases for optimal NF-kB activation (Viatour et al., 2005). Upon stimulation by TNF-α, activation of the IKK complex leads also to phosphorylation of p65. Parallel with the activation of NF-kB, TNF-α can also induce the translocation of NF-kB dimers from the cytoplasm to the nucleus. This is called nuclear translocation of NF-kB (Ferrari et al., 2017; Viatour et al., 2005).

The effect of TNF-α on the activation of NF-kB was determined in multiple studies (Ferrari et al., 2017; Jia et al., 2015; Kadioglu et al., 2015; Piegholdt et al., 2014; Speciale et al., 2013). For example, Ferrari et al. (2017) confirmed that Caco-2 cells exposed to TNF-α had increased levels of the p65 NF-kB subfraction in nuclear extracts of the cells. In the nucleus, the activated NF-kB dimers bind to the target genes and regulate their transcription leading to the expression of many pro-inflammatory mediators. As a possible result of the TNF-α induced activation of NF-κB, *in vitro* models observed that TNF-α induced stress resulted in an increased production of the chemokines MCP-1, IL-8, IL-6 by EA.hy926 cells (Jia et al., 2015), HUVEC (Jia et al., 2013) and Caco-2 cells (Peng et al., 2017) and an enhanced production of the adhesion molecules ICAM-1 and VCAM-1 by endothelial cells (Ferrari et al., 2017; Jia et al., 2013; Jia et al., 2015). In line with this observation, it was reported that treatment of HUVEC cells and EA.hy926 cells with TNF-α increases the attraction and firm adhesion of monocytes to endothelial cells (Chanet et al., 2013; Del Bo' et al., 2016; Jia et al., 2013).
Moreover, it has been reported multiple times that TNF-α stimulates the production of ROS in multiple cell types (Corda et al. , 2001; García-Ruiz et al., 1997; Goossens et al., 1995; Nishikawa et al., 2007; Serra et al., 2016). Speciale et al. (2013) showed that exposure of HUVEC cells to TNF-α reduced the intracellular levels of the endogenous antioxidant enzyme SOD compared to unexposed cells, which led

to the conclusion that TNF-α treatment resulted in a redox imbalance towards a more oxidized state. Furthermore, TNF-α also accounts for reduced junctional protein levels and activation of proteinases that cleave into the extracellular matrix which both contribute to a TNF-α dependent loss of transepithelial electrical resistance (TEER) and an increase in monolayer permeability in intestinal epithelial cells such as Caco-2 (Cremonini et al., 2017) and HT-29/B6 cells (Amasheh et al., 2012) and endothelial cells such as HUVEC and EA.hy926 cells (McKenzie & Ridley, 2007).

An important mechanism that plays a role in the cellular defense against oxidative stress is the activation of the nuclear factor-like 2 (Nrf2) signaling pathway. This signaling pathway controls the expression of genes whose protein products are involved in the detoxication and elimination of reactive oxidant species (Nguyen et al., 2009). When it comes to the activation of the Nrf2 signaling pathway by TNF-α, the results are different depending on the cell line: according to Serra et al. (2016) stimulation of HT-29 intestinal cells with TNF-α significantly increases Nrf2 DNA binding activity compared to the levels in untreated cells up to 8 h of cytokine challenge. However, after 16 h of cytokine exposure, DNA binding activity of Nrf2 was similar to that of non-stimulated cells. On the other hand, Speciale et al. (2013) concluded that TNF-α does not significantly affect nuclear translocation of Nrf2 in HUVEC endothelial cells.

#### 1.2.1.2.2 Lipopolysaccharides (LPS)

A second way to induce inflammation *in vitro* is with an exogenous mediator such as lipopolysaccharides (LPS). LPS are large molecules that occur in the outer membrane of gram-negative bacteria and consist out of a lipid and a polysaccharide (Raetz & Whitfield, 2002). They are endotoxins, which cause a strong immune response initiated by the interaction of LPS with receptors for microbial products, such as Toll-like receptors (TLR) (Raetz & Whitfield, 2002). The best-studied member of this family of receptors, involved in the recognition of LPS, is TLR-4 (Panaro et al., 2012). This group of transmembrane proteins are located on the surface of many cell types including monocytes such as THP-1, dendritic cells and macrophages (Cani et al., 2007) but also intestinal epithelial cells (Cario et al., 2000) and endothelial cells (Dauphinee & Karsan, 2006).

It has been described that the interaction of LPS with TLR-4 results in the activation of the transcription factor NF-κB (Cho et al., 2016; J. H. Kim et al., 2015; Lee et al., 2016; Ma et al., 2015; Wang et al., 2014; Y. Zhou et al., 2018). Activation of the NF-κB pathway by LPS induces the expression and release of a broad array of inflammatory mediators including TNF-α in HUVEC cells (Lee et al., 2014; Ma et al., 2015; Shalini et al., 2016), RAW 246.7 cells (Hou et al., 2015; J. H. Kim et al., 2015) and THP-1 cells (di Gesso et al., 2015). This way, stimulation of cells with LPS gives rise to the same biomarkers of inflammation as seen with TNF-α induced inflammation including an increase in IL-1β and IL-6 by endothelial cells (Ma et al., 2015; Y. Zhou et al., 2018) and leukocytes (di Gesso et al., 2015; Hou et al., 2015), an increase in IL-8 levels by epithelial cells (S. Lee et al., 2016), enhanced levels of adhesion molecules ICAM-1, VCAM-1 and E-selectin by endothelial cells (Cho et al., 2016; W. Lee et al., 2015; Shalini et al., 2016) and increased barrier permeability of endothelial cells which subsequently promote

monocyte adhesion to the endothelium (Huang et al., 2015). Furthermore, LPS induces a decrease in anti-inflammatory cytokine IL-10 by leukocyte cells RAW 264.7 (Hou et al., 2015) and THP-1 (di Gesso et al., 2015).

In addition, it has been reported that LPS not only activates the NF-κB pathway but also the mitogen-activated protein kinase (MAPK) pathway by the phosphorylation of MAP kinases (ERK, p38 and JNK) in HUVEC cells (Bae, 2015; Ma et al., 2015) and RAW 264.7 cells (Ishisaka et al., 2013). Moreover, studies show an increase in oxidative stress after LPS treatment caused by elevated ROS levels (C. Li, Zhang, & Frei, 2016) and increased NO production in epithelial cells (S. Lee et al., 2016), endothelial cells (Y. Zhou et al., 2018) and leukocytes (S. H. Kim et al., 2015; Park & Song, 2013; R. Wang et al., 2016).

## 1.2.2 Inflammation and mitochondrial dysfunction

### 1.2.2.1 The connection between mitochondrial dysfunction and the inflammatory pathway

The predominant physiological function of mitochondria is the generation of adenosine triphosphate (ATP) by oxidative phosphorylation (M. D. Brand & Nicholls, 2011). However, they also have many additional functions including the generation and detoxification of reactive oxygen species (ROS), and the regulation of calcium homeostasis and apoptosis. Abnormalities in any of these processes can be termed as mitochondrial dysfunction (M. D. Brand & Nicholls, 2011). Mitochondrial dysfunction can directly or indirectly be initiated by a multitude of processes including pathogen infection, aging, hyperglycemia, lipotoxicity and exposure to environmental toxins (West, 2017).

An important indication of mitochondrial dysfunction is an increase in the production of ROS by the mitochondrial respiratory chain, also referred to as mitochondrial ROS (mtROS), during oxidative phosphorylation (West, 2017). Leakage of electrons from the electron transport chain can lead to the partial reduction of oxygen to form superoxide (Carrasco-Pozo et al., 2012). Subsequently, superoxide is converted into hydrogen peroxide by two dismutases including superoxide dismutase 2 (SOD2) in the mitochondrial matrix and superoxide dismutase 1 (SOD1) in the mitochondrial intermembrane space. Collectively, both superoxide and hydrogen peroxide are considered as mtROS (X. Li et al., 2017).

Physiological levels of mtROS participate as “redox messengers” in intracellular signaling and regulation. In this scenario, a small change in mtROS levels may be repressed by the endogenous antioxidant system involving antioxidative defense enzymes such as catalase and glutathione peroxidase, oxidant scavengers such as vitamin C, vitamin E and carotenoids and other mechanisms that repair oxidant-induced damage to macromolecules (López-Armada et al., 2013). However, an important cause and effect of mitochondrial dysfunction is an increase in the net production of mitochondrial mtROS caused by an uncontrolled generation of ROS which exceeds the capacity of the cellular antioxidant system (Chung et al., 2009; Hsu et al., 2013).

As discussed in section 1.2.1.1., (mt)ROS can induce an inflammatory response by both upregulating transcription factors and creating (mt)DAMPs. In turn, there is also extensive evidence that inflammatory mediators may induce mitochondrial dysfunction, this way promoting a dangerous inflammatory cycle. **Figure 4** gives a simplified schematic overview of the interaction between the inflammatory pathway and mitochondrial dysfunction.

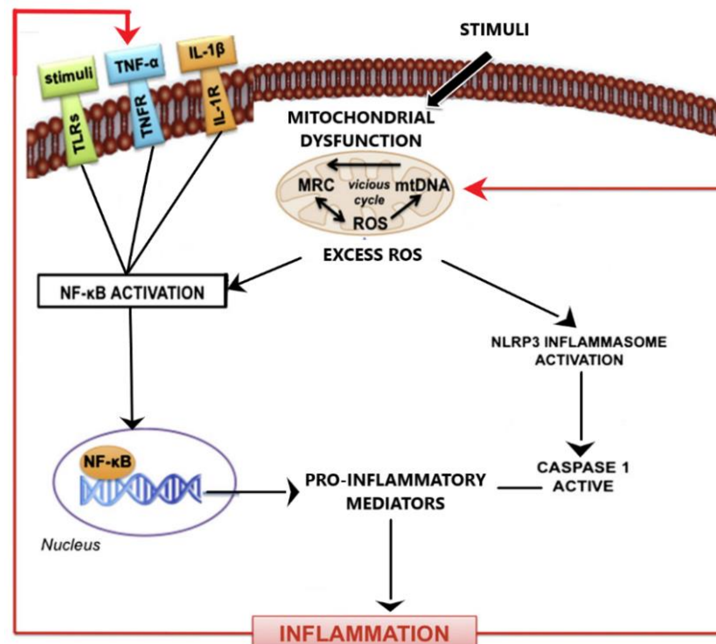


*Figure 4: Theoretical model for the interrelation between mitochondrial dysfunction and the inflammatory response (Based on López-Armada et al., 2013).*

For example, an excess of (mt)ROS, due to mitochondrial dysfunction, activates redox-sensitive inflammatory transcription factors such as NF-κB, which result in a subsequent increase in the expression of cytokines, chemokines and adhesion molecules (Lopez-Armada et al., 2013). Furthermore, an increase in oxidative stress levels due to dysfunctional mitochondria can damage membrane lipids, proteins and mitochondrial DNA. In turn, this may lead to the disruption of the mitochondrial membrane integrity and consequently to the release of mitochondrial DAMPs (mtDAMPS) such as mtROS and mtDNA into the cytosol or extracellular environment (West, 2017). These molecules engage with pattern recognition receptors (PRRs) of the immune system which in turn, modulate the immune response via redox-sensitive inflammatory pathways or by activation of the NLRP3 (nucleotide-binding domain, leucine-rich repeat pyrin domain-containing-3) inflammasome (López-Armada et al., 2013). Inflammasomes are cytosolic molecular complexes formed by NLRs, which are responsible for the activation of caspase-1 and -5 and subsequently the proteolytic activation of the proinflammatory cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18) (Abderrazak et al., 2015). While all inflammasomes recognize certain pathogens, the NLRP3 inflammasome can be activated by non-microbiological stimuli including (mt)ROS (Nakahira et al., 2011; R. Zhou, Yazdi, Menu, & Tschopp, 2011).

In turn, there is also extensive evidence that a great number of inflammatory mediators, such as TNF-α, IL-1β and NO, may induce mitochondrial dysfunction (Blaser et al., 2016). For example, J. Kim et al. (2010) showed that the mitochondrial function in human chondrocytes was disturbed after treatment with pro-inflammatory cytokines IL-1β and TNF-α via induced mitochondrial DNA damage and decreased ATP production. In addition, the production of mitochondrial mtROS was enhanced following cytokine exposure. Blaser et al. (2016) also reported that TNF can mediate mitochondrial ROS production and this way, activate NF-κB.

#### 1.2.2.2 Assessing mitochondrial dysfunction *in vitro*

Usually, when one wants to assess mitochondrial (dys)function, one investigates the ability of mitochondria to produce ATP appropriately in response to energy demands. This process is highly depending on the mitochondrial proton circuit: three electron transport complexes, I, III and IV, but not complex II, which is only involved in the transport of electrons from succinate to the quinone pool (Q), pump protons across the mitochondrial inner membrane during oxidative phosphorylation (Zorova et al., 2018). A drop in redox potential, linked to the electrons passing through the complex, is in each case coupled to the expulsion of protons from the mitochondrial matrix into the intermembrane space. Thereafter, protons re-enter the matrix through ATP synthase during active ATP synthesis. This proton circuit creates a difference in electrical potential across the inner membrane, also called mitochondrial membrane potential ($\Delta\psi_m$) and a pH gradient between the intermembrane space and the mitochondrial matrix (ΔpH). Those two components together form the protonmotive force which is harnessed to produce ATP (M. D. Brand & Nicholls, 2011; Zorova et al., 2018). Furthermore, in addition to proton re-entry through ATP synthase, mitochondria also possess a parallel endogenous proton leak in order to limit the protonmotive force. The protonmotive force needs to be restricted, for example when the ATP synthesis is blocked, in order to prevent dielectric breakdown of the membrane and to restrict leakage of single electrons from the electron transport chain (M. D. Brand & Nicholls, 2011).

Mitochondrial (dys)function can be assessed in isolated mitochondria, in intact cells (*in vitro*) or *in vivo* (M. D. Brand & Nicholls, 2011). In case of in *vitro* studies, a wide range of stressors can be applied to disrupt the ATP production pathway in order to induce mitochondrial dysfunction. In this study we focused on the stressors oligomycin, FCCP and valinomycin. These stressors are normally not present in the *in vivo* circulation, but are interesting to use in mechanistic *in vitro* studies since they disrupt different parts of the proton motive circuit and subsequently the ATP production (Moore & Pressman, 1964; Penefsky, 1985).

Oligomycin belongs to the group of macrolides commonly produced by *Streptomyces* (Han et al., 2015). The component is known to inhibit mitochondrial ATP-synthase which is necessary for oxidative phosphorylation of ADP to ATP (Zorova et al., 2018). It is proposed that oligomycin inhibits ATPase activity by causing a conformational change in the F0 portion of the complex that is transmitted to F1, resulting in an impaired binding of substrate in the catalytic sites. According to W. Zhou et al. (2018), oligomycin can permeate cell membranes and mitochondrial membranes and subsequently reach ATP-

synthase because of its strong amphipathic character. Furthermore, the inhibition of ATP synthase by oligomycin results in a slight mitochondrial hyperpolarization and subsequently an increase in proton leakage (Zorova et al., 2018). Since oligomycin blocks mitochondrial ATP production, it is associated with a shift towards glycolysis. Although most cell lines have sufficient glycolytic capacity to allow this, in some cells oligomycin can induce an ATP deficiency. A shortage of ATP may disrupt various reactions that require ATP, leading to a failure in the supply of substrate and a progressive decrease in respiration (Zorova et al., 2018). For example, Poór et al. (2014) showed a reduction in ATP levels of Madin-Darby Canine Kidney (MDCK) cells and an increase in hyperpolarization of the mitochondria upon oligomycin treatment. According to a study by Giovannini et al. (2002), treatment of intestinal Caco-2 cells with oligomycin caused an increase in mitochondrial membrane potential.

A second way to disrupt ATP synthesis is by using the protonophoric, uncoupling agent FCCP which transports protons through the mitochondrial membrane, before they can be used to provide the energy for oxidative phosphorylation. In this way, FCCP induces a drop in both ΔΨm and ΔpH and it disconnects the electron transport chain from the formation of ATP (Benz & McLaughlin, 1983). The electron transport chain will respond to the drop in $\Delta\Psi_m$ and ΔpH with an increase in respiration (Zorova et al., 2018). Therefore, the respiration rate after addition of FCCP is also called the maximum respiration rate. Moreover, it is worth mentioning that FCCP acts as a protonophores across all membranes, this way acidifying the cytosolic compartment (Zorova et al., 2018).

Finally, one can also use an ionophore such as valinomycin to induce mitochondrial dysfunction. Valinomycin is structurally and functionally similar to the bacterial toxin cereulide, a toxin produced by *Bacillus cereus* which is associated with food poisoning (Makarasen, Yoza, & Isobe, 2009; Tempelaars, Rodrigues, & Abee, 2011). Valinomycin is a toxic cyclic dodecadepsipeptide which has a central hydrophilic cavity which accommodates potassium ions, while the external surface possesses hydrophobic chains (Gonzales et al., 2016). This enables valinomycin to form a lipid-soluble complex with $K^+$, which is abundantly present in the cytosol. Whereas $K^+$ penetrates the hydrophobic membrane only very slowly, the lipid-soluble $K^+$-valinomycin complex readily passes through the hydrophobic cell membrane (Gonzales, Smagghe, et al., 2016). This causes an influx of positive ions through the inner mitochondrial membrane, which neutralizes the excess of negative charge inside the matrix, diminishing the mitochondrial membrane potential ($\Delta\psi_m$) but not ΔpH, in contrast to FCCP. Thus, valinomycin slows down mitochondrial ATP synthesis without blocking electron transfer to $O_2$ (Moore & Pressman, 1964). Valinomycin not only drives $K^+$ ions inside the mitochondrial matrix but it also enables to transport potassium ions extracellularly, causing hyperpolarization of the cell membrane (Gonzales, Smagghe, et al., 2016; Moore & Pressman, 1964). According to Gonzales, Smagghe, et al. (2016), treatment of Caco-2 cells with valinomycin induces also a significant increases in ROS production.

## 1.3 Impact of flavonoids on cellular stress

### 1.3.1 *In vitro* models used to study the impact of flavonoids on cellular stress

*In vitro* models can be used as inexpensive, fast, screening tools for research on potential bioactives and they are ideal for mechanistic research. The disadvantage is that most of these models do not take into account the different interactions between the different tissues ("cross-talk"), which can affect the results.

#### 1.3.1.1 Intestinal epithelial cells

The intestinal epithelium is a highly organized cellular system consisting out of proliferating stem cells located at the base of the intestinal crypts and differentiated intestinal epithelial cell types (e.g. enterocyte) at the top of the villi, all maintained in a dynamic steady state (Van Der Flier & Clevers, 2009). A well-established and widely used model of the human intestinal barrier is the human colon carcinoma cell line (Caco-2) (Gonzales, Smagghe, et al., 2016). The differentiation process of Caco-2 cells *in vitro* reflects the maturation process of the cells *in vivo* (Hidalgo et al., 1989). Furthermore, differentiated Caco-2 cells express functional tight junctions, brush border characteristics and morphological and functional characteristics of mature enterocytes (Hidalgo et al., 1989). However, several reports suggested that the results, when using this parental cell line, are not reproducible because a lack in homogeneity (Gonzales et al., 2016). To improve the reproducibility, clones such as TC-7 were isolated from the late passage of parental Caco-2 cells to get a more homogenous cell line. Reportedly, the differences in paracellular transport and passive diffusion properties are small between these clones and the parental Caco-2 cell line. However, P-glycoprotein mediated active efflux of cyclosporin was found to be higher in TC7 cells compared with the Caco-2 parental line which makes the TC7 cell lines, according to Turco et al. (2011), a less suitable model in studies that evaluate flavonoid intestinal transport. Other intestinal epithelial cell lines such as the human colon cancer cell line HCT 116, are more frequently used to study cancer biology since this cell line is a highly tumorigenic and growth factor-independent (Christensen et al., 2012; Rajput et al., 2008).

#### 1.3.1.2 Endothelial cells

The wall of all human blood vessels is lined by an thin single layer of endothelial cells, the endothelium (Alberts, 2017). These cells play important roles in blood pressure regulation, adhesion and transmigration of leukocytes from the vessel to the target tissue, blood coagulation and the formation of new blood vessels (angiogenesis) (Bouïs et al., 2001). In the past, many *in vitro* studies used human umbilical vein endothelial cells (HUVEC) since these cells form a monolayer similar to the endothelial cells *in vivo* (Del Bo' et al., 2016; Gonzales et al., 2015). However, according to a review by Bouïs et al. (2001) are HUVEC cells not ideal to study endothelial processes because of their short lifespan and different behavior depending on their origin. The authors recommend EA.hy926 cells if large-vessel endothelium is to be investigated.

### 1.3.2 The impact of flavonoids on cellular stress

Flavonoids are first and foremost known because of their antioxidant capacities as a way to reduce oxidative stress. The mechanism behind the antioxidant activity of flavonoids is mainly linked to the free radical scavenging capacity of the hydroxyl substituents primarily on the B-ring (Heim et al., 2002). These hydroxyl groups donate hydrogen and an electron to hydroxyl, peroxyl, and peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoid radical. Furthermore, flavonoids have chelating properties which also contribute to their antioxidant activity because of the direct relation between hydroxyl radical production and the concentration of copper and iron (Heim et al., 2002; H. Zhang & Tsao, 2016). However, dietary flavonoid concentrations in the cytosol are much lower than the concentration of endogenous antioxidants such as vitamin C and glutathione in the cytosol (Stevens et al., 2018). Therefore, it is unlikely that their health promoting effects *in vivo* are based only on their direct free radical scavenging and chelating properties. As a result, more recent studies have demonstrated that the bioactivity of flavonoids is linked with their interaction with cell signaling pathways (Stevens et al., 2018). This way, flavonoids can indirectly suppress oxidative stress by reducing inflammatory responses and increasing endogenous antioxidant pathways. Moreover, a few studies even suggested a direct effect of flavonoids on mitochondrial function. This multidimensional effect is likely responsible for the overall consistent effectiveness of flavonoids although the mechanism behind many of these effects is still not fully understood (Heim et al., 2002; H. Zhang & Tsao, 2016).

As part of this study, we reviewed *in vitro* studies from the past five years (2013-2018) which determined the influence of flavonoids on cellular stress in various cell lines. We focused on studies that used TNF-α and LPS to induce the inflammatory pathway and the mitochondrial stressors oligomycin, FCCP and valinomycin to induce direct mitochondrial dysfunction. An overview of the different studies can be found in the appendix. The results of these studies are discussed in following sections.

#### 1.3.2.1 Effect of flavonoids on biomarkers of cellular stress induced by TNF-α

**Appendix 1** gives an overview of the most recent in vitro studies (2013-2018) on the effect of flavonoids on cellular stress induced by TNF-α. According to many of those studies, flavonoids such as luteolin (0,5-20μM) (Jia et al., 2015) and cyanidin-3-O-glucoside (20-40μM) (Ferrari et al., 2017; Ferrari et al., 2016; Speciale et al., 2013) inhibit TNF-α-induced IκBα degradation, NF-κB p65 nuclear translocation and subsequent NF-κB transcriptional activity in both intestinal and endothelial cells. This ability to inhibit the NF-κB pathway may explain why addition of those flavonoids to TNF-α-stressed intestinal and endothelial cells also decreased the levels of inflammatory biomarkers (IL-8, IL-6) (Ferrari et al., 2017; Ferrari et al., 2016; Speciale et al., 2013) and the expression of adhesion molecules (ICAM-1, VCAM-1, E-Selectin) (Ferrari et al., 2017; Jia et al., 2015) and chemokines (Jia et al., 2015) which critically regulate the adherence of monocytes to the cells. This way, flavonoids luteolin, genistein, naringenin, cyanidin, malvidin, delphinidin and their corresponding metabolites inhibited TNF-α-induced binding of monocytes to endothelial cells (both HUVECs and EA.hy926) (Del Bo' et al., 2016; Jia et al., 2013; Jia et al., 2015). In addition, it is interesting to mention that Jia et al. (2013) determined that while pretreatment of genistein reduced the biomarkers of inflammation such as the production of adhesion

molecules and chemokines by HUVEC cells and the adhesion of monocytes to the endothelial cells, post-treatment of genistein failed to suppress TNF-α-induced adhesion of monocytes to HUVECs. These results indicate that the moment of flavonoid exposure may influence their protective properties against TNF-α-induced endothelial inflammation.

Besides the effect of flavonoids on the NF-κB pathway, it has also been reported that flavonoid administration may decrease TNF-α-induced permeability in intestinal cells, including proliferating Caco-2 (Piegholdt et al., 2014), differentiated Caco-2 cells (Cremonini et al., 2017) and HT-29 intestinal cells (Amasheh et al., 2012). For example, Cremonini et al. (2017) established that O-glucosides of cyanidin and delphinidin protected the monolayer of differentiated Caco-2 intestinal cells from TNF-α-induced decrease of TEER and increase of FITC-dextran permeability.

Furthermore, multiple studies investigated also the influence of flavonoids on the TNF-α induced changes in redox status. As mentioned before, oxidative stress is involved in the modulation of TNF-α-induced-inflammation. Serra et al. (2016) observed, using TNF-α treated HT-29 cells, that the flavonoids cyanidin-3-glucoside and resveratrol induced a decrease in ROS production and elevated GSH/GSSG ratios. Also, Ferrari et al. (2017) showed that pretreatment of Caco-2 cells with cyanidin-3-glucoside (C3G) caused a protective effect against the changes in the cellular oxidative status exerted by TNF-α. Interestingly, C3G pretreatment increased GSH levels even in absence of external stimuli, demonstrating that C3G is able to induce an adaptive response (Ferrari et al., 2017). Using endothelial HUVEC cells, Speciale et al. (2013) showed a reduction in oxidative stress levels when the cells were treated with cyanidin-3-O-glucoside. Moreover, the reports by Serra et al. (2016) and Speciale et al. (2013) both documented a significant upregulation of the Nrf2 pathway after pretreatment of the cells. According to the researchers, activation of the Nrf2 pathway by flavonoids, which induces antioxidant mechanisms, may be one of the molecular mechanisms responsible for blocking the TNF-α induced activation of NF-kB (Speciale et al., 2013). On the other hand, Jia et al. (2013) concluded that the flavonoid genistein has no significant effect on various antioxidant enzyme activities in the endothelial HUVEC cells and it does not scavenge free radicals. Thus, their results indicate that the activity of genistein on TNF-α-induced endothelial dysfunction is not mediated through an antioxidant mechanism. In conclusion, many of these studies support the hypothesis that the anti-inflammatory effect of flavonoids on TNF-α-induced cellular stress in different cell lines is linked to decreasing the NF-κB pathway.

#### 1.3.2.2 Effect of flavonoids on biomarkers of cellular stress induced by LPS

**Appendix 2** gives an overview of the most recent in vitro studies (2013-2018) on the effect of flavonoids on cellular stress induced by LPS. First, these studies demonstrated that flavonoids can suppress LPS-induced secretion of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in the monocytic cell line RAW264.7 (Hou et al., 2015; Nishitani et al., 2013; G. Yang et al., 2013), in endothelial HUVEC cells (W. Lee et al., 2014; Ma et al., 2015; Shalini et al., 2016; W. Zhou et al., 2018), intestinal Caco-2 cells (Nishitani et al., 2013) and even HEK293 kidney cells (X. Zhang et al., 2014). According to di Gesso et

al. (2015) decreases the secretion of TNF-α in LPS-stimulated THP-1 cells when the cells were treated with different flavonoids, but the secretion of IL-1β and IL-10 did not significantly change. Furthermore, the flavonoid apigenin prevented LPS-induced decrease of anti-inflammatory cytokine IL-10 in kidney cells (X. Zhang et al., 2014) and the flavonoid dihydromyricetin had the same effect in RAW264.7 cells (Hou et al., 2015). Moreover, quercetin 3,7-O-dimethyl decreased the production of IL-8 in HT-29 intestinal cells (S.-g. Lee et al., 2016). In addition, flavonoids are also able to inhibit the LPS-induced expression of cell adhesion molecules (CAMs) such as ICAM-1, VCAM-1 and E-selectin by endothelial cells (Cho et al., 2016; S.-g. Lee et al., 2016; C. Li, Zhang, & Frei, 2016; Shalini et al., 2016). Also, a decrease in LPS-induced monocyte adhesion and transendothelial migration of leukocytes, such as THP-1 monocytes, to human endothelial cells has been reported upon flavonoid treatment (Cho et al., 2016; W. Lee et al., 2014).

Furthermore, flavonoids such as wogonin can increase the amount of junction proteins in HUVECs, this way protecting the barrier integrity upon LPS stress (Huang et al., 2015). According to various research papers, flavonoids also inhibited pro-inflammatory enzyme activities, such as COX-2 (J. H. Kim et al., 2015; S. Lee et al., 2016; Park & Song, 2013; R. Wang et al., 2016) and iNOS (S. Lee et al., 2016; R. Wang et al., 2016; T. Wang et al., 2014; Y. Zhou et al., 2018). The decrease in iNOS and COX-2 activity consequential decreased the production of nitric oxide (NO) (J. H. Kim et al., 2015; R. Wang et al., 2016) and PGE2 (S. H. Kim et al., 2015; Park & Song, 2013).It was also determined that addition of quercetin (5-20µM) results in the decrease of LPS-induced ROS content in human aortic endothelial (HAEC) cells (C. Li, Zhang, & Frei, 2016).

Various studies stated that the suppressive effect of flavonoids, including prunetin (G. Yang et al., 2013), orientin (W. Lee et al., 2014), luteolin (Park & Song, 2013), resveratrol (Panaro et al., 2012), fisetin (J. H. Kim et al., 2015) and baicalin (Wonhwa Lee et al., 2015), on the biomarkers of cellular stress is due to the inhibition of LPS-induced NF-κB activation in Caco-2 cells (Panaro et al., 2012), HUVEC cells (W. Lee et al., 2014; Wonhwa Lee et al., 2015) and RAW 246.7 (J. H. Kim et al., 2015; Park & Song, 2013; G. Yang et al., 2013) as shown by decreases in p65 nuclear translocation, p65 phosphorylation and nuclear factor NF-κB. For example, Nishitani et al. (2013) studied the intestinal anti-inflammatory activity of luteolin in a co-culture setup of macrophages and intestinal epithelial cells. They concluded that luteolin inhibits NF-κB nuclear translocation in the macrophages, followed by reduction of TNF-α, IL-1β and IL-6 mRNA expression, which also resulted in the downregulation of IL-8 mRNA expression in the intestinal cells (Nishitani et al., 2013). Moreover, in LPS stressed HUVEC cells, Cho et al. (2016) observed a decreased NF-kB activity when cells are exposed to quercetin.
On the other hand, other research studies did not show a significant reduction in NF-κB reporter activity in cells treated with flavonoids. For example, di Gesso et al. (2015) studied the effect of different flavonoids (quercetin, naringenin, hesperetin) and their metabolites on LPS-induced NF-κB reporter activity in THP-1 cells. Their results showed no significant reduction in NF-κB reporter activity. Furthermore, C. Li, Zhang, and Frei (2016) reported that the flavonoid quercetin did not inhibit the activation of NF-κB in LPS-induced human aortic endothelial cells (HAEC). They suggested that the

inhibitory effect of quercetin on the biomarkers of inflammation is not due to inhibition of NF-κB activation, but instead due to the activation of the Nrf2 pathway, which results in gene transcription and protein expression of the antioxidant enzymes, which in turn inhibits LPS-induced formation of oxidants (ROS). Finally, a study by Ishisaka et al. (2013) confirmed that quercetin did not affect the nuclear translocation nor phosphorylation of NF-κB in the LPS-stimulated RAW 264 cells.
Overall, these contradictory findings suggest that the anti-inflammatory pathway of flavonoids may differ depending on the flavonoid and the cell line. Moreover, Park and Song (2013) reported that luteolin decreases LPS-induced inflammation in RAW 264 cells better than luteolin-7-O-glucoside which suggest that the metabolism of the flavonoids also has an effect on their capacity to reduce inflammation.

Furthermore, studies show that flavonoids can also inhibit the LPS-induced MAPK signaling pathway by inhibiting the activation of MAP kinases (Ishisaka et al., 2013; Wonhwa Lee et al., 2015; Ma et al., 2015). Therefore, some studies suggested that the inhibition of the NF-kB signaling pathway and MAPK signaling pathway could partially be explained by the capacity of flavonoids to decrease the LPS-induced expression of TLR-4 protein levels as seen in Caco-2 cells (T. Wang et al., 2014) and HUVECs (Huang et al., 2015; Y.-q. Zhou et al., 2018).

#### 1.3.2.3 Effect of flavonoids on mitochondrial function and mitochondrial dysfunction

**Appendix 3** gives an overview of the most recent in vitro studies (2013-2018) on the effect of flavonoids on mitochondrial dysfunction. Even though there is a clear link between inflammation and mitochondrial dysfunction as discussed in section 1.2.2., mechanistic research on the effect of flavonoids on mitochondrial dysfunction, induced by specific mitochondrial modulators (such as oligomycin, FCCP and valinomycin), is still scarce. However, there are some more recent studies that review the effect of flavonoids on mitochondrial function in unstressed cells. Bernatoniene and Kopustinskiene (2018) gives an overview of the effects of catechins on mitochondrial functions, such as increased ATP production, reduction in ROS production, uncoupling of respiration, suppression of anaerobic glycolysis, enhanced phosphorylation at lower concentrations but also inhibition of the respiratory chain at higher concentration. Moreover, de Oliveira et al. (2016) reviews the capacity of quercetin on modulating pathways associated with mitochondrial biogenesis, oxidative respiration, ATP generation, intra-mitochondrial redox status and mitochondrial membrane potential. They referred to studies demonstrating the ability of quercetin to inhibit ATPase (Dorta et al., 2005; Lang & Racker, 1974). However, the concentrations of quercetin used in those *in vitro* models were very high and may not be reached in *in vivo* situations (de Oliveira et al., 2016). Furthermore, de Oliveira et al. (2016) also suggested that quercetin may affect the redox parameters related to the mitochondria via methods involving its chemical structure and/or its direct action on components of the mitochondrial electron transport chain for example by modulation of the complex I activity, an important source of peroxide. It is suggested that the structural similarity of quercetin with the quinone moiety of coenzyme Q results in the ability of quercetin to bind to the binding site of complex I (Murphy, 2009; Van Acker et al., 1996). In addition, they also referred to a study by Lagoa et al., (2011) that showed that quercetin (10 μM), kaempferol and apigenin, have the ability to inhibit complex I in brain submitochondrial particles without

changing the activity of complexes II and III, suggesting that quercetin has the ability to associate to the binding site of the complex.

A first study that investigated the influence of flavonoids on mitochondrial dysfunction induced by mitochondrial stressors is a study by Carrasco-Pozo et al. (2012). They studied the protective effect of quercetin, resveratrol and rutin on Caco-2 cells towards indomethacin-induced mitochondrial dysfunction. Indomethacin inhibits the activity of mitochondrial complex I which leads to the accumulation of NADH, the overproduction of mitochondrial peroxide, decreased ATP levels and lower mitochondrial membrane potential. Their findings suggested that flavonoids, especially quercetin, had a protective role against mitochondrial dysfunction complementary to their antioxidant property. The researchers hypothesized that the structural similarity of quercetin with rotenone may favor the binding of quercetin to the ubiquinone site of complex I, protecting it from inhibitors, such as indomethacin or rotenone. On the other hand, there are some contradictory findings concerning this topic which led to the conclusion that quercetin has a potent capacity to modulate mitochondrial function however the mechanisms behind it are not fully understood. Moreover, a study published by Lagoa et al. (2011) determined that quercetin and kaempferol had the ability to counteract increased mitochondrial $H_2O_2$ production in isolated mitochondria from rat brain and heart, in the presence of rotenone and antimycin A which are inhibitors of the respiratory chain. The same study also determined that treatment of the same isolated mitochondria with quercetin, kaempferol and apigenin up to 10 µM did not affect the rate of oxygen consumption but did inhibit complex I activity which led them also to the conclusion that complex I may be the major target of quercetin and kaempferol for the inhibition of mtROS production.
In addition, Gonzales et al. (2016) demonstrated that co-administration of quercetin and valinomycin to undifferentiated Caco-2 cells, resulted in a significant decrease of intracellular ROS levels, compared to cells treated with only valinomycin. They also demonstrated a change in localization, cellular uptake and metabolism of quercetin in undifferentiated Caco-2 cells caused by valinomycin. According to Nisha et al. (2014) are quercetin and apigenin able to decrease the depolarization of the mitochondrial membrane potential of adipocytes upon tunicamycin stress although the exact mechanism remains again unclear. On the other hand, Poór et al. (2014) reported that the flavonoid diosmetin had no effect on the reduced ATP concentrations induced by oligomycin in MDCK kidney cells.

In conclusion, there is supporting evidence that flavonoids influence the biomarkers of mitochondrial dysfunction but more research is needed to better understand the mechanisms behind it.

# 2 PROBLEM STATEMENT AND OBJECTIVES

As mentioned in the literature review, the activity of dietary flavonoids may play a role in the prevention of a broad range of diseases including cancers and cardiovascular diseases. Because of their large bio-active potential, it is not surprising that flavonoids receive a lot of attention from consumers and the food industry. Consequently, a lot of research in this field has been done and is still being done *in vivo* and *in vitro*. Up until now, many *in vitro* studies have demonstrated the beneficial effects of flavonoids on cellular stress levels in different cell lines as described in section 1.3.2. However, despite the fact that flavonoids are able to reduce cellular stress, related to chronic diseases, it is also known that flavonoids have a rather poor bioavailability (section 1.1.2). This conundrum is also referred to as the "flavonoid paradox" (section 1.1.3). Finding an explanation for this paradox is critical in understanding the mechanism behind the health promoting effects of flavonoids. However, studies in literature around this topic are still scarce.

Based on what we know from studies in literature, we suggest that the cell environment to which the cells are exposed under healthy and stressed conditions could alter their response to the bioactive flavonoids. This bring us to the general hypothesis of this master dissertation: can cellular stress alter the intracellular flavonoid accumulation, resulting in higher in situ flavonoid concentrations in the stressed cells, which may counteract cellular stress in the cells? In this master dissertation we focus on the most common dietary flavonoid: quercetin.

The first objective of this master dissertation is to investigate the impact of cellular stress on the intracellular quercetin accumulation in intestinal and endothelial cell lines. Based on the close interaction between the inflammation pathway and mitochondrial dysfunction (as discussed in section 1.2.2), two forms of cellular stress will be induced: (i) inflammatory stress (both host-related and microbial-related) and (ii) mitochondrial stress. In case of the latter, different complexes of the electron transport chain will be targeted.

Based on the results of part one, the second objective of this research is to investigate the influence of quercetin on cellular stress levels and mitochondrial function in intestinal cells, using a range of high throughput bioassays.

# 3 MATERIALS AND METHODS

## 3.1 Reagents and chemicals

The human colorectal carcinoma cell line Caco-2 (HTB-37™), the human endothelial cell line EA.hy926 (CRL-2922™) and HCT 116 (CCL-247™) were obtained from American type culture collection (ATCC). High glucose DMEM cell culture medium, trypsin and sterile phosphate buffered saline (PBS) were purchased from Life Technologies. Dimethylsulfoxide (DMSO), trisaminomethane (Tris) buffer, paraformaldehyde, trichloroacetate 2-aminoethyl-diphenylborinate (DPBA), penicillin/streptomycin (P/S), non-essential amino acids (NEAA), non-sterile PBS tablets, oligomycin, valinomycin, FCCP, LPS, TNF-α, ascorbic acid, Sulforhodamine B, trichloroacetic acid, Bradford reagent, BSA, resazurin, Triton X-100, ethanolamine, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), N-acetyl cysteine, formaldehyde, EDTA, o-phtalaldehyde, tetramethylrhodamine, ethyl ester (TMRE), glutamine, pyruvate, glucose and quercetin were all bought from Sigma-Aldrich. Trypan blue and fetal bovine serum (FBS) were obtained from Amresco and Greine bio-one, respectively. Ethanolamine was purchased from Janssens Chimica and glacial acetic acid was obtained from Fisher Scientific. The Seahorse XF Cell Mito Stress kit, XF-base medium and XF calibrant solution were all obtained from Agilent. And finally, the luminescent ATP Detection Assay Kit were bought from Abcam.

## 3.2 Cell culture

Three commercially available cell lines from human origin were used: intestinal cells (Caco-2 and HCT 116) and endothelial cells (EA.hy926). Each cell line was cultivated in Dulbecco's Modified Eagle's Medium (DMEM) containing GlutaMAX™, 4.5 g/l D-glucose and pyruvate supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% non-essential amino acids (NEAA). The cells were grown in T75 polystyrene cell culture flasks in an incubator at 37 °C in a humidified atmosphere with 10% $CO_2$.

## 3.3 Experimental setup

### 3.3.1 Seeding of cells in plates

For the analyses, cells were seeded in transparent (cell viability assay, glutathione, ATP assay) or black (intracellular accumulation assay, ROS assay, mitochondrial membrane potential assay) 96-well plates with a density of 60 000 cells per cm² of growth area in growth cell culture medium. For the Seahorse assay, cells were seeded in Agilent Seahorse 24-well XF Cell Culture Microplate at a density of 100 000 cells/cm² also in FBS-containing medium. Counting of the cells happened with the Bürker counting chamber using the Trypan blue method.

### 3.3.2 Exposure of cells to stressor and/or quercetin

Proliferating Caco-2, HCT 116 and EA.hy926 cells were exposed to the compounds in FBS-free medium 24h post-seeding. Differentiated Caco-2 cells were exposed 21 days post-seeding. Quiescent EA.hy926 cells were obtained by starving (FBS-free and NEAA-free cell culture medium) the cells for 24h, six days after seeding, and were subsequently exposed to the compounds of interest in FBS-free medium.

**Table 2** gives an overview of the cell lines and stressors that were used to induce cellular stress for each assay. Inflammatory stress was induced by TNF-α or LPS. TNF-α is an endogenous stressor and will simulate a host induced inflammatory response. Moreover, the exogenous stressor LPS, which is present in the outer membrane of Gram-negative bacteria, will also induce an pro-inflammatory environment (Panaro et al., 2012). Furthermore, mitochondrial stress is induced by mitochondrial stressors oligomycin, FCCP or valinomycin. Each of these theoretical stressor will target different the electron transport chain in a different way to induce mitochondrial dysfunction. More information about the mode of action of each stressor can be found in section 1.2.2.2.

*Table 2: Cell lines and stressors (with concentrations) used during the treatment step in each assay. Each concentration of each stressor was also applied in co-administration with 10 µM quercetin (in case of Caco-2 and HCT 116 cells) or 3 µM (in case of EA.hy926 cells).*

| Assay | Cell line(s) | Stressors used to induce cellular stress in each cell line + concentration | |
|---|---|---|---|
| **Cell viability assay** | Proliferating Caco-2<br>Differentiated Caco-2<br>HCT 116<br>Proliferating EA.hy926<br>Quiescent EA.hy926 | oligomycin<br>FCCP<br>valinomycin | 100 ng/ml and 1 µg/ml<br>1 ng/ml and 500 ng/ml<br>0.1 ng/ml and 1 ng/ml and 5 ng/ml |
| **Intracellular accumulation quercetin (part 1)** | Proliferating Caco-2<br>Differentiated Caco-2<br>HCT 116<br>Proliferating EA.hy926<br>Quiescent EA.hy926 | oligomycin<br>FCCP<br>valinomycin | 100 ng/ml and 1 µg/ml<br>1 ng/ml and 500 ng/ml<br>0.5 ng/ml and 1 ng/ml and 5 ng/ml |
| **Intracellular accumulation quercetin (part 2)** | Proliferating Caco-2 | LPS<br>TNF-α | 1 ng/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml<br>1 ng/ml, 5 ng/ml, 10 ng/ml |
| **ROS levels** | Proliferating Caco-2 | oligomycin<br>FCCP<br>valinomycin | 100 ng/ml and 1 µg/ml<br>1 ng/ml and 500 ng/ml<br>0.1 ng/ml and 1 ng/ml and 5 ng/ml |
| **Glutathione** | Proliferating Caco-2 | oligomycin<br>FCCP<br>valinomycin | 100 ng/ml and 1 µg/ml<br>1 ng/ml and 500 ng/ml<br>0.1 ng/ml and 1 ng/ml and 5 ng/ml |
| **Mitochondrial membrane potential (MMP)** | Proliferating Caco-2 | oligomycin<br>FCCP<br>valinomycin | 100 ng/ml and 1 µg/ml<br>1 ng/ml and 500 ng/ml<br>0.1 ng/ml and 1 ng/ml and 5 ng/ml |
| **Seahorse** | Proliferating Caco-2 | oligomycin<br>FCCP<br>valinomycin | 100 ng/ml<br>1 ng/ml<br>0.1 ng/ml |
| **ATP levels** | Proliferating Caco-2 | oligomycin<br>FCCP<br>valinomycin | 100 ng/ml and 1 µg/ml<br>1 ng/ml and 500 ng/ml<br>0.1 ng/ml and 1 ng/ml and 5 ng/ml |

For each experiment, the cells were exposed to these stressors in those concentrations with or without quercetin in co-administration. In case of a co-administration with quercetin, the Caco-2 and HCT 116 cells were exposed to 10 µM quercetin and the EA.hy926 cells to 3 µM quercetin, since previous studies showed that 10 µM quercetin may induce toxicity in the EA.hy926 cells. These concentrations also represent the *in vivo* situation better since intestinal cells come into contact with higher flavonoid concentrations than the endothelial cells.

It is important to mention that in case of a co-administration with quercetin, a pretreatment of 1 hour with quercetin in DMEM FBS-free exposure medium (without the stressor) was performed before treatment. Furthermore, in each experiment, three to six biological replicates where treated with FBS-free medium, and three to six biological replicates where treated with 3 or 10 µM quercetin in FBS-free medium, both to serve as a reference. One well of one multiwell plate represent one biological replicate. The cells were in each of the experiments exposed to the treatment for 24 hours at 37°C and $CO_2$-concentration of 10 % until the actual bioassay.

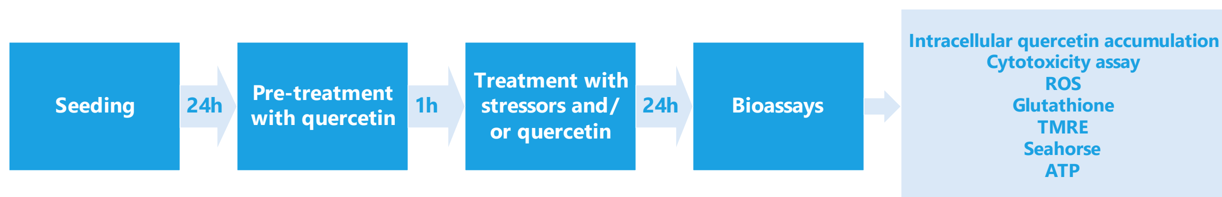


*Figure 5: Timeframe experiments*

## 3.4 Bioassays

### 3.4.1 Determination of protein content

To measure the cellular protein content in each well, the widely established **SRB** (Sulforhodamine B) assay was used. The SRB assay relies on the property of Sulforhodamine B (SRB) to bind to amino acid residues of proteins in the cells (Papazisis, Geromichalos, Dimitriadis, & Kortsaris, 1997). The amount of bound SRB is then a proxy for the amount of cells in each well. The cellular protein content was measured at the end of each experiment to correct the data for the cell content in each well.
Briefly, the cell monolayers were first fixed with 50 µL of 50% TCA (trichloroacetic acid in MilliQ®-water), at 4°C for at least 1 hour. Subsequently, the plate was washed 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 repeatably rinsed with 1% glacial acetic acid in MilliQ®-water to remove the excess stain after which 200 µL of 10mM Tris-buffer (tris(hydroxymethyl)aminomethane) was added and suspended until the protein-bound dye was completely dissolved in the buffer. Absorbance was determined at 490 nm using the Spectramax M2 Multimode Plate Reader.

In addition, to estimate the number of cells in a lysate, we used the **Bradford assay.** First, 5 µL of the unknown protein samples were transported to a 96-well plate after which 250 µL Bradford reagent was added. This reagent contains Brilliant Blue G dye in phosphoric acid and methanol. The protein-dye

complex that is formed, causes a shift in the absorption maximum of the dye to 595 nm. The absorbance was measured at 595 nm after 30 minutes using the Spectramax M2 Multimode Plate Reader. In addition, a BSA standard curve was established in order to quantify the measurements for the protein concentration using a series of known concentrations of BSA (Sigma) protein between 0 mg/mL and 1.4 mg/mL. The Bradford assay was performed on each of these dilutions which resulted in a standard curve showing the absorbance in function of the protein concentration (mg/mL).

### 3.4.2 Cell viability assay

To establish the relative cytotoxicity of the stressors oligomycin, valinomycin and FCCP, we used a cell viability assay based on the properties of resazurin (7-Hydroxy-3H-phenoxazin-3-one-10-oxide). Resazurin is a cell-permeable compound that is blue in color and virtually non-fluorescent. Upon entering cells, viable cells with active metabolism who maintain a reducing environment within their cytosol, with usually NADPH or NADH as the reductant, reduce resazurin by dehydrogenase enzymes such as NADPH dehydrogenase or NADH dehydrogenase, to form the resorufin product which is pink and highly fluorescent (**Figure 6**). This way, the quantity of resorufin produced is proportional to the metabolic activity in the cells and subsequently the viability of the cells (Candeias et al., 1998; Riss et al., 2016).

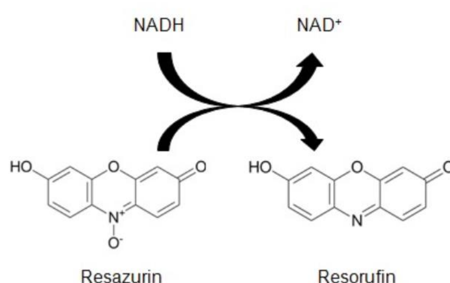


*Figure 6: Resazurin (non-fluorescent) is reduced, in the presence of NADPH dehydrogenase or NADH dehydrogenase as the enzyme, with NADPH or NADH as the reductant, to resorufin (highly fluorescent) (Riss et al., 2016)*

For this experiment, the exposure medium used to treat the cells was supplemented with sodium ascorbate (1 µM) to stabilize quercetin. Briefly, the cells were exposed to 10 µg/ml resazurin for 2h at 37°C and 10% $CO_2$. As a reference for the background fluorescence were some of the wells also treated with 10 µg/ml resazurin. After this incubation step, the supernatants of each well was transferred to a black 96-well plate and the fluorescence was measured (λex/em=560/590 nm). Subsequently, an SRB assay was performed as described above on the same plates to know the protein content of each well.

### 3.4.3 Intracellular accumulation of quercetin

The intracellular accumulation of quercetin in cellular stress induced cells was determined with a spectrofluorimetric method that used 2-aminoethyl-diphenylborinate (DPBA). It is generally known that quercetin, like most other flavonoids, is poorly fluorescent in aqueous solutions, but exhibits an increased fluorescence when it forms a spontaneous complex with specific probes such as 2-

aminoethoxydiphenyl borate (DPBA) (J. H. Lee et al., 2014). For this assay the exposure medium was supplemented with sodium ascorbate (1 µM). Briefly, after the 24h exposure, the cells were gently washed with 200µl of phosphate buffered saline (PBS) and then fixed by adding paraformaldehyde (4% in PBS). The plates with the paraformaldehyde were stored for 12h at 4°C. After the fixation step, cells were washed with 10 mM ethanolamine and subsequently with PBS. Thereafter, the cells were permeabilized with Triton-X100 (0.5% in PBS), stained with 2-aminoethyl-diphenylborinate (0.2% w/v in 0,3% dimethyl sulfoxide (DMSO) in water) and incubated for 2h at 37°C. To correct for the background fluorescence, one replicate of each condition was not stained, but just treated with 0.3% DMSO in water. The fluorescence ($\lambda_{ex/em}$=485/520 nm) was measured using the Spectramax M2 Multimode Plate Reader. Finally, an SRB assay was performed, as described above, on the same plates to determine the protein content in each well.

### 3.4.4 Determining ROS levels

The get an idea of the oxidative stress level in the cells, the reactive oxygen species (ROS) content in the cells was determined using oxidant-sensing fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCF-DA) (Eruslanov & Kusmartsev, 2010). $H_2$DCF-DA is a nonpolar dye which will transport across the cellular membrane and is then converted into the polar nonfluorescent derivative dichlorodihydrofluorescein ($H_2$DCF) by cellular esterases. Subsequently, intracellular ROS and other peroxides will oxidize the compound into the highly fluorescent dichlorofluorescein (DCF) (**Figure 7**) (Canvax, 2019).

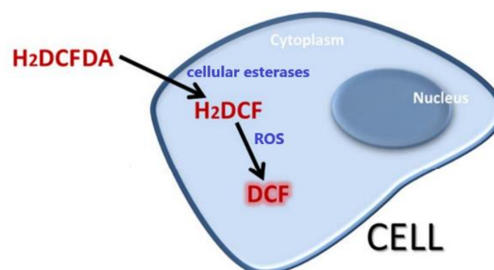


*Figure 7: Mechanism behind determination of intracellular ROS levels using $H_2$DCF-DA (Canvax, 2019)*

During the assay, the exposure medium was first removed from the black 96 well plate and 200 µL of 20mM $H_2$DCF-DA was added in each well. The plate was then incubated for 30 minutes at 37°C and 10% $CO_2$. After 30 minutes, the stain was removed, followed by the addition of 200 µL PBS. The fluorescence was measured ($\lambda_{ex/em}$=485/535 nm) using the Spectramax M2 Multimode Plate Reader. An SRB assay was performed, as described above, to determine the protein content in each well.
The determination of the ROS levels in the Caco-2 cells cell was performed two separate times. The first time was the assay performed after a short time exposure period of 4 hours. A second assay was performed on Caco-2 cells that were exposed to the treatment during a long term exposure period of 24 hours.

## 3.4.5 Glutathione assay

Glutathione (GSH) is an antioxidant and is capable of preventing damage to important cellular components caused by reactive oxygen species. In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent ($H^{+}$+ $e^{-}$) to other molecules, such as reactive oxygen species to neutralize them. After donating an electron, glutathione itself becomes reactive and readily reacts with another reactive glutathione to form glutathione disulfide (GSSG). Once oxidized, glutathione can be reduced back by glutathione reductase, using NADPH as an electron donor. The ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) within cells is often used as a measure of cellular oxidative stress.

To determine GSH and GSSG levels o-phthalaldehyde (OPT) was used as a fluorescent reagent. The method takes advantage of the reaction of GSH with OPT at pH 8 and the reaction of GSSG with OPT at pH 12. N-ethylmaleimide (NEM) has been used to prevent auto-oxidation of GSH during measurement of GSSG and EDTA (ethylenediaminetetraacetic acid) is added to the samples to avoid bivalent metal binding (**Figure 8**) (Hissin & Hilf, 1976; Singh et al., 2017).

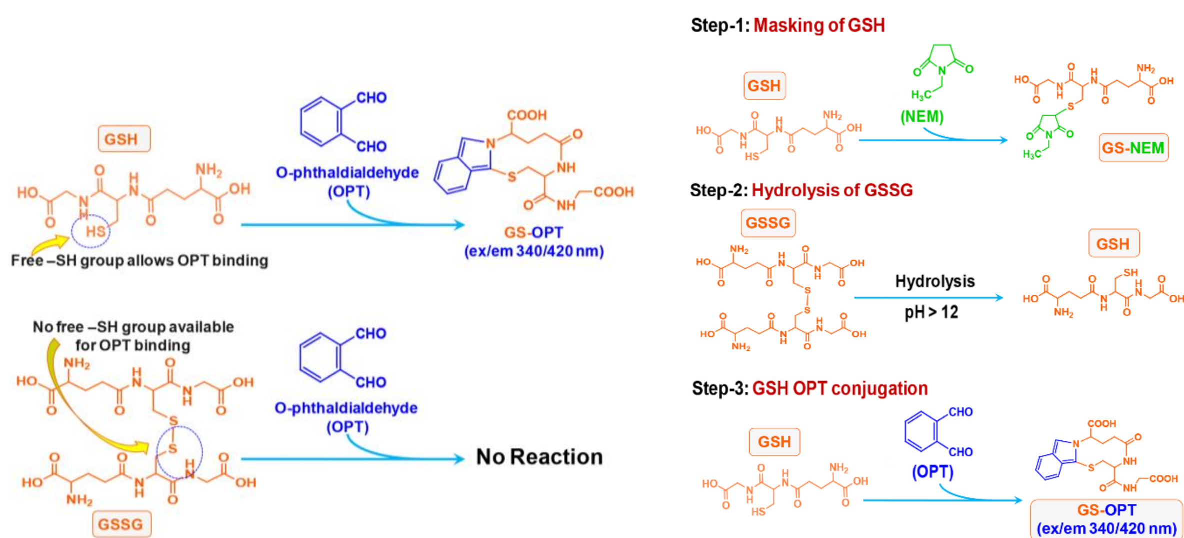


*Figure 8: Mechanism behind the glutathione assay (Singh et al., 2017).*

During the assay, the cells were washed with PBS and homogenized with 200 µL of 20 mM Tris solution with 0.1% Triton X100. For the GSH quantification, the plate was kept on ice during the assay to avoid oxidation. First, 10 µL of the cell homogenate was transferred to a new black 96 well plate and 10 µL buffered formaldehyde (37-40% formaldehyde/ 0.1 M phosphate buffer, pH 8 in a 1/4 ratio) was added and incubated at room temperature for 5 minutes. Next, 170 µL phosphate-EDTA buffer (phosphate buffer 0.1 M – 5 mM EDTA (ethylenediaminetetraacetic acid), pH 8) and 10 µL OPT (o-phtalaldehyde, 1 mg/mL in absolute methanol) were added of 45 minutes after which the excitation at 355 nm and emission at 460 nm was recorded with the Spectramax M2 Multimode Plate Reader. For the GSSG quantification, 10 µL of the cell homogenate was transferred to a new black well plate to which 10 µL buffered formaldehyde was added. In addition, 4 µL of N-ethylmaleimide (NEM) was added after 5 minutes, to prevent auto-oxidation of GSH during measurement of GSSG. The plate was then

incubated for 30 minutes at room temperature after which 10 µL OPT and 176 µL of 0.1 NaOH (to increase the pH > 12) were added. The plate was incubated for 45 minutes at room temperature before the fluorescence at an excitation wavelength of 355 nm and emission wavelength of 460 nm was measured.

### 3.4.6 Mitochondrial membrane potential assay

The mitochondrial membrane potential was measured using tetramethylrhodamine, ethyl ester (TMRE) which is a cell permeant, positively-charged, red-orange dye. This dye accumulates within mitochondria in inverse proportion to $\Delta\Psi_m$ according to the Nernst equation. The dye accumulates in inverse proportion to $\Delta\Psi_m$ so the more negative the $\Delta\Psi_m$, the more TMRE dye will accumulate in the mitochondria and the higher the fluorescent signal will be (**Figure 9**). Fluorescent dye accumulation in mitochondria is optically detected by the fluorescent plate reader. Active mitochondria have a relative negative charge so more TMRE will accumulate in those then in depolarized or inactive mitochondria who have decreased membrane potential and fail to sequester TMRE (Scaduto Jr & Grotyohann, 1999).

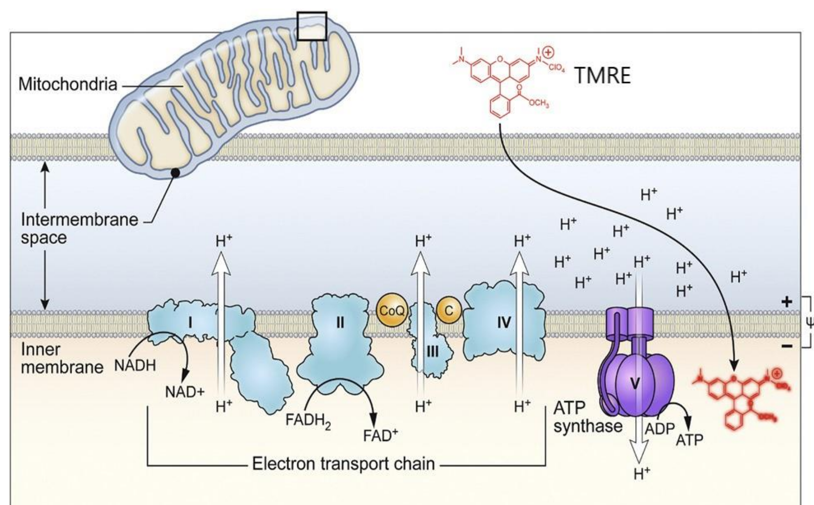


*Figure 9: TMRE mechanism (Sukumar et al., 2016).*

For this assay, were the treatment components diluted in phenol red free FBS-free medium used to reduce interference. After the 24h treatment, the medium was removed and phenol red free FBS-free medium containing 1 µM of TMRE was added to the cells. The plate was incubated for 20 minutes at 37 °C. Next, the plate was washed two times with 0.2% solution of BSA in PBS. Fluorescence reading was performed at Ex/Em = 549/575 nm using the Spectramax M2 Multimode Plate Reader. An SRB assay was performed to determine the protein content in each well. As a positive control and negative control oligomycin (50 µg/ml) and FCCP (5 µg/ml), respectively, were added to untreated cells 30 min (for oligomycin) and 10 min (for FCCP) before the assay was performed.

### 3.4.7 Measuring mitochondrial respiration using a Seahorse assay

The XF24 Analyzer (Seahorse) measures the aerobic mitochondrial respiration ($O_2$ consumption), or anaerobic energy production after glycolysis in the cytosol by measuring the pH decrease, using a probe

based system. The system can automatically inject modulators during the course of the Seahorse-run to investigate basal respiration, as well as changes in the respiration after injection of certain modulators. During this assay, the Agilent Seahorse XF Cell Mito Stress Test was used which contains modulators of the electron transport chain such as oligomycin, FCCP and a mix of rotenone and antimycin A. During the assay, the oxygen consumption rate (OCR) and pH (ECAR) is measured before and after the addition of each modulator to derive several parameters of mitochondrial respiration. A typical response of cells after exposure to these compounds is depicted in **Figure 10**.

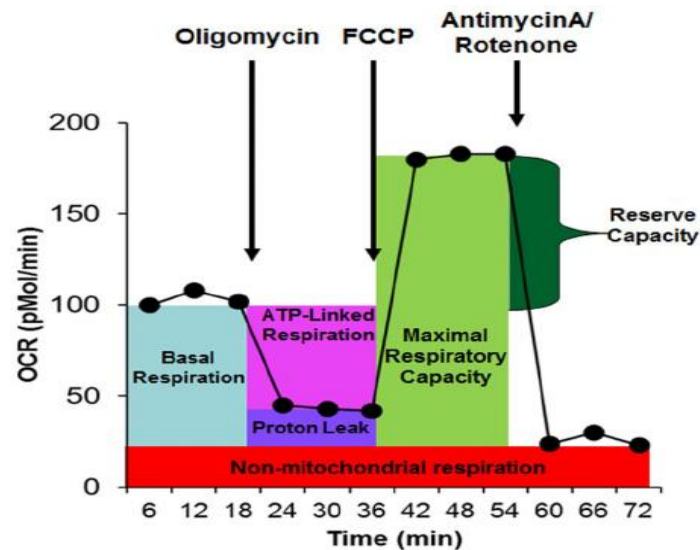


*Figure 10: Typical respiration curve during Seahorse assay. Oxygen consumption rate (OCR) is measured before and after the addition of inhibitors to derive several parameters of mitochondrial respiration (Agilent, 2019).*

Initially, baseline cellular OCR is measured, from which basal respiration can be derived by subtracting non-mitochondrial respiration. Next, oligomycin, an inhibitor of ATP synthase that disrupts this way oxidative phosphorylation, is injected and the measured OCR is used to derive ATP-linked respiration (by subtracting the oligomycin rate from baseline cellular OCR) and proton leak respiration (by subtracting non-mitochondrial respiration from the oligomycin rate). Secondly, FCCP is injected which is an uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential allowing the electron transport chain (ETC) to function at its maximal rate. Based on the results of the FCCP induced OCR rate, the maximal respiratory capacity is derived. Finally, antimycin A and rotenone, inhibitors of complex III and I, are added during the third injection, to shut down ETC function, revealing the non-mitochondrial respiration driven by processes outside the mitochondria. **Figure 11** illustrates the complexes of the Electron Transport Chain (ETC), and indicates the target of action of all the modulators included in this assay.

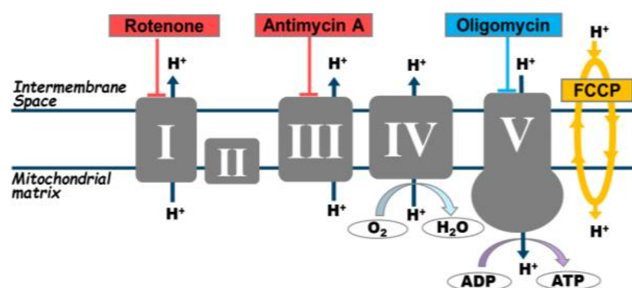


*Figure 11: The target of action for all the modulators included in the Seahorse XF Cell Mito Stress Test Kit (Agilent, 2019).*

In this experiment, we wanted to investigate the effect of a 24h pretreatment with the different stressors oligomycin, valinomycin and FCCP with or without quercetin in co-administration, on the aerobic mitochondrial respiration. Five biological replicates of each treatment condition were randomly spread over two 24 well plates. The XF24 probe-containing cartridge was hydrated overnight in XF calibrant solution, at 37°C under normal air conditions, in a plastic bag with wet tissue to avoid evaporation of the calibrant solution. The day of the assay, 24 hours after addition of the pretreatment, non-buffered and glucose-free XF-base medium was supplemented with 2 mM glutamine, 1 mM pyruvate and 10mM glucose and the pH was adjusted to 7.4 at 37°C. Next, 500 µL of this medium was loaded to each well and the cells were then incubated for 1 hour, at 37°C under normal air conditions. In the meantime, modulators of the MitoStress Kit, including oligomycin, FCCP and antimycin/rotenone were transferred to the three injection ports of the hydrated sensor cartridge of each well. After the calibration of the probes, the oxygen consumption rate was measured before the addition of the modulators, followed by measurements after the injection of oligomycin, FCCP and antimycin/rotenone. The concentrations of each modulator after sequential injection in the wells during the assay was 1 µM for oligomycin, 0.25 µM for FCCP and 0.5 µM of antimycin/rotenone. The oxygen consumption (OCR) and extracellular acidification (ECAR) for the basal situation and after injection of each modulator, were measured 3 times during 4 minutes, with 4 minutes interval between each measurement. After analysis, an SRB test was performed to normalize the response to cell density.

In addition, to determine the acute effect of quercetin on the mitochondrial respiration, a second seahorse assay was performed on untreated Caco-2 cells by first injecting 1 µM oligomycin and then a second injection of quercetin using different concentrations (2µM – 25µM). An SRB assay was performed after analysis.

### 3.4.8 ATP assay

Total levels of cellular ATP (adenosine triphosphate) were determined using the luminescent ATP Detection Assay Kit. The ATP assay is based on the production of light caused by the reaction of ATP with added firefly's luciferase and luciferin. The emitted light is proportional to the ATP concentration inside the cell. The reaction can be summarized as follows:

First, 50 µL of the detergent was added into each well to lyse the cells. The wells were homogenized after which 50 µL of Substrate Solution was added to each of the wells. Next, each well was again homogenized and the plate was then covered for 10 minutes to adapt the plate to the dark. Finally the luminescence was measured using the Spectramax M2 Multimode Plate Reader. In addition, a standard curve was made to quantify the results. The kit inactivates ATPases (ATP degrading enzymes) during the lysis step, ensuring that the luminescent signal obtained truly corresponds to the levels of cellular ATP. At the end, a Bradford assay was performed to correct the ATP results for protein content in each well.

## 3.5 Statistics

All data is corrected for the cell mass in each well. The data shown in the figures below represent mean values ± the standard deviation. Differences among the data of the different concentration groups were statistically analyzed. All data, with exception of the accumulation data, was analyzed by SPSS Statistics 25 software by one-way analysis of variance (ANOVA) methods. A post hoc tukey test was used when the variance was not significantly different and dunnett post hoc when variance were different. $p < 0.05$ was regarded as statistical significance. The accumulation data was statistically analyzed by the t-test in excel.

# 4 RESULTS

## 4.1 Determining cytotoxicity of stressors for different cell lines

In this master dissertation the influence of quercetin on cellular stress, induced by the application of (i) oligomycin, (ii) FCCP, (iii) valinomycin, (iv) TNF-α and (v) LPS, was studied. The first step was the investigation of the cytotoxicity of all these stressors in order to avoid severe, acute toxicity. The concentrations of valinomycin, TNF-α and LPS were already optimized before for the intestinal (Caco-2 and HCT 116) and endothelial (EA.hy926) cells (Vissenaekens et al., submitted). However, the effect of oligomycin and FCCP on cellular activity still had to be determined. The stressor concentrations that were tested were based on concentrations that are frequently used in literature studies (Duluc et al., 2014). The results for each cell line are depicted in **Figure 12**.

First, the results of the cytotoxicity assay showed that the metabolic activity of the proliferating intestinal (Caco-2 and HCT 116) and endothelial (EA.hy926) cells were not altered by the tested concentrations of oligomycin and FCCP with or without quercetin in co-administration. The same observation was made for the differentiated Caco-2 and quiescent EA.hy926 cells.

Furthermore, it is worth mentioning that treatment of all cell lines did not significantly change the protein content in the cells compared to the untreated cells ($p < 0,05$; see SRB data in appendix).

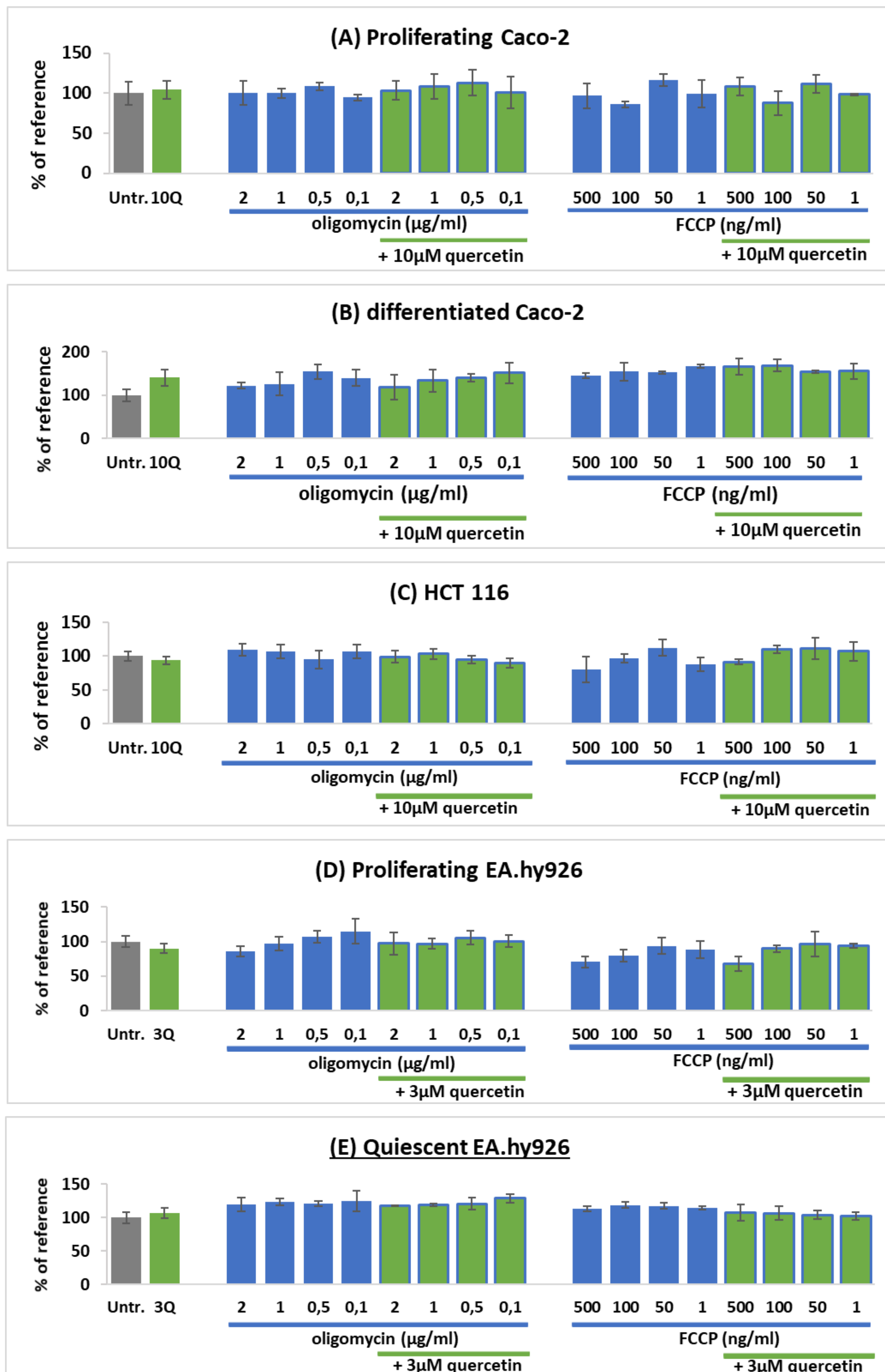


*Figure 12: Metabolic activity of the cells. The results are corrected with the SRB data for cell count and are displayed as the average of the 6 biological replicates relative to the untreated cells. Error bars indicate the standard deviation of biological replicates (N= 6, for the untreated and 10µM quercetin conditions; N=3 for other conditions). *, **, *** denote statistical difference with p<0.05, p<0.01 and p<0.001, respectively. (Untr. = untreated cells; 10Q = 10 µM quercetin and 3Q = 3 µM quercetin)*

## 4.2 Intracellular accumulation of quercetin in intact stressed cells

Next, it is hypothesized that cellular stress may alter flavonoid uptake, metabolism and accumulation as mentioned in section 1.1.3 of the literature study. Therefore, we investigated the intracellular accumulation of quercetin in intestinal and endothelial cell lines under stressed conditions. Cellular stress was induced in the different cell types by mitochondrial stressors (i) oligomycin, (ii) FCCP and (iii) valinomycin or inflammatory stressors such as (i) LPS or (ii) TNF-α.

### 4.2.1 Intracellular quercetin accumulation in stressed cells induced by mitochondrial stressors

First, the influence of cellular stress induced by the mitochondrial stressors (i) oligomycin and (ii) FCCP on the intracellular accumulation of quercetin in five different cell lines (proliferating Caco-2, differentiated Caco-2, HCT 116, proliferating EA.hy926 and quiescent EA.hy926) was investigated. In case of the proliferating Caco-2 cells, a third mitochondrial stressor was investigated: valinomycin. The result of the accumulation assay for mitochondrial stressors are shown in **Figure 13.**

For the proliferating Caco-2 cells, the quercetin accumulation increased significantly with the addition of the stressor oligomycin (1 µg/ml and 100 ng/ml), FCCP (500 ng/ml and 1 ng/ml) and valinomycin (1 ng/ml), compared to the non-stressed cells. The accumulation of quercetin in the stressed Caco-2 cells was up to 15 times higher than the accumulation in the stressed cells (fig. 13A). The increase in quercetin accumulation upon valinomycin stress was limited to 50% compared to non-stressed cells, which is significantly less than the effects seen upon oligomycin and FCCP exposure (fig. 13B). The intracellular quercetin accumulation did not significantly increase with the addition of either of the stressors for the differentiated Caco-2 cells (fig. 13C) or the HCT 116 cells (fig. 13D). .

In proliferating EA.hy926 cells, quercetin accumulation increased up to 40% when the cells were stressed with 100 ng/ml oligomycin; FCCP did not induce significant changes (fig. 13E). On the other hand, our results of the accumulation assay for quiescent EA.hy926 cells showed that oligomycin did not induce changes in intracellular quercetin accumulation but exposure to FCCP stress resulted in an increase in quercetin accumulation, compared to non-stressed cells (fig. 13F).

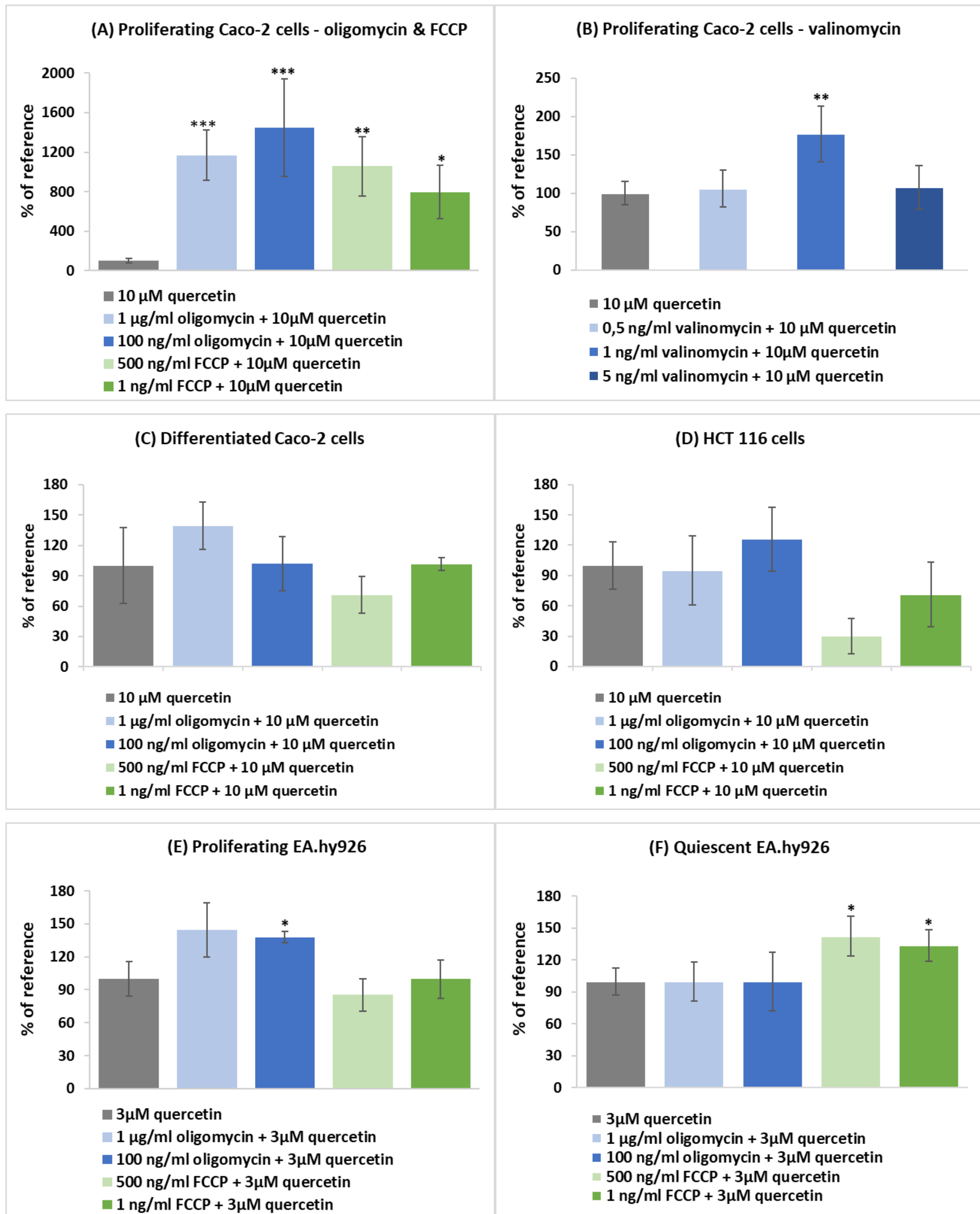


*Figure 13: Mean intracellular flavonoid accumulation under the influence of oligomycin and FCCP. Error bars indicate SD of biological replicates (N=6). *, **, *** denote statistical difference with p<0.05, p<0.01 and p<0.001, respectively.*

### 4.2.2 Intracellular quercetin accumulation in stressed Caco-2 cells induced by inflammatory stressors

Based on the result we had seen for proliferating Caco-2 cells stressed with oligomycin, FCCP and valinomycin, we also investigated the accumulation of quercetin in the same cell line stressed with inflammatory stressors LPS and TNF-α (**Figure 14**).

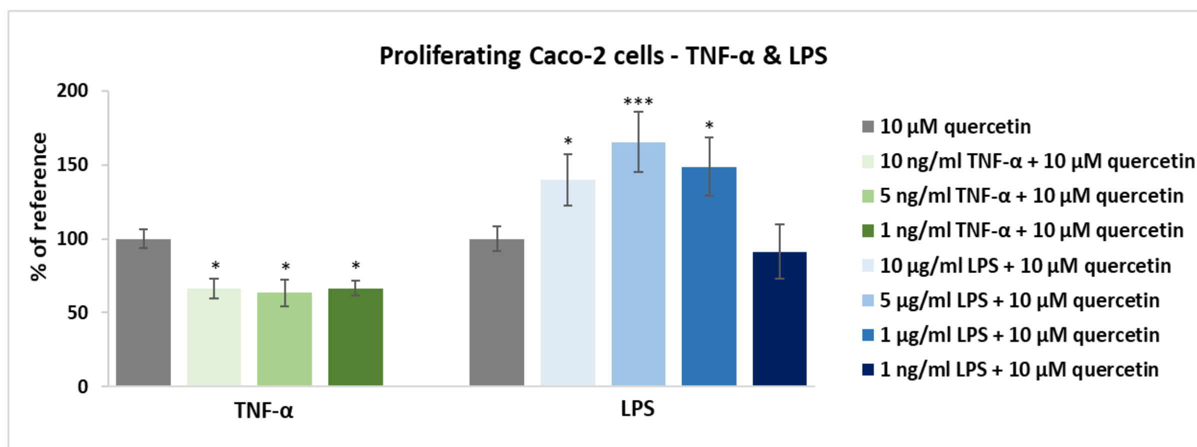


*Figure 14: Mean intracellular flavonoid accumulation in proliferating Caco-2 cells under the influence of stressors TNF-α (left) and LPS (right). Error bars indicate SD of biological replicates (N=6). *, **, *** denote statistical difference to the reference (non-stressed cells) with $p<0.05$, $p<0.01$ and $p<0.001$, respectively.*

In case of proliferating Caco-2 cells stressed with TNF-α, there was not a significant increase in intracellular quercetin accumulation but a significant decrease for all tested concentrations. On the other hand, exposure of the proliferating Caco-2 cells to LPS (> 1 ng/ml) resulted in a significant increase in quercetin accumulation.

## 4.3 Effect of quercetin on oxidative stress levels in Caco-2 cells

Above described experiments showed that quercetin accumulation is altered upon cellular stress, especially for proliferating Caco-2 cells stressed with the mitochondrial stressors (i) oligomycin, (ii) FCCP and (iii) valinomycin. Oxidative stress is an important type of stress that is associated with mitochondrial dysfunction as described in section 1.2.2.1 of the literature review. We suspected that mitochondrial dysfunction induced by the stressors (i) oligomycin, (ii) FCCP and (iii) valinomycin contributes to the excessive formation of reactive oxygen species. Therefore, we wanted to investigate if the accumulated quercetin may alter oxidative stress levels in the cell.

### 4.3.1 ROS levels

To determine the effect of quercetin on the oxidative stress levels in proliferating Caco-2 cells in which cellular stress is induced by oligomycin, FCCP or valinomycin, ROS levels were determined by a spectrofluorometric method. The assay was performed after the proliferating Caco-2 cells were exposed to these stressors with or without 10 µM quercetin in co-administration, for a short time period (4h) and

a long term exposure period (24h). The results of the ROS assay after the short exposure period (4h) and long exposure period (24h) are depicted in **Figure 15A** and **Figure 15B**, respectively.

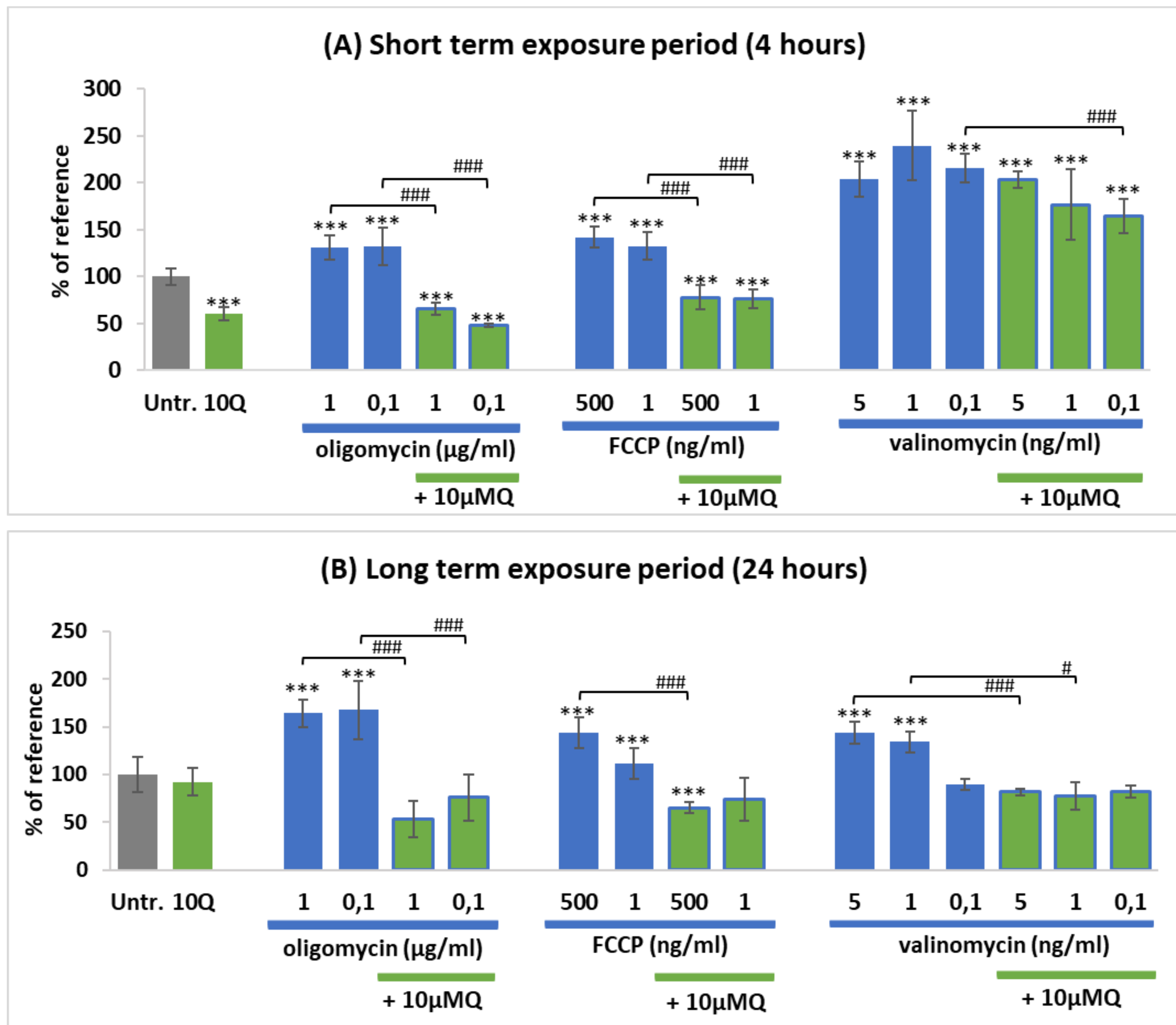


*Figure 15: Mean ROS/SRB relative to untreated cells in proliferating Caco-2 cells. Error bars indicate SD of biological replicates (N=6). *, **, *** denote statistical difference compared to the values for the untreated cells (reference) with p<0.05, p<0.01 and p<0.001, respectively. #, ##, ### denote statistical difference between stressed and non-stressed conditions with p<0.05, p<0.01 and p<0.001, respectively. (Untr. = untreated cells; 10Q = 10µM quercetin)*

Our results depicted in figure 15 showed that 24h exposure of the Caco-2 cells to 10 µM quercetin did not decrease the ROS levels in the cells compared to the untreated cells (fig. 15B). On the other hand, exposure of the proliferating Caco-2 cells to 10 µM quercetin for 4 hours significantly decreased the amount of ROS per unit of cell mass compared to the untreated cells with around 40% (fig. 15A). This could be due to the direct antioxidant capacity of quercetin.

Exposure of the cells to oligomycin, FCCP or valinomycin increased the amount of ROS in the proliferating Caco-2 significantly compared to the untreated cells for both short and long term exposure. So inducing mitochondrial stress in the proliferating Caco-2 cells with oligomycin, FCCP or valinomycin significantly increased the amount of reactive oxygen species in the cells as expected. Interestingly,

when the cells were exposed to the same stressors but in co-administration with 10 µM quercetin, the ROS levels in the cells were significantly decreased compared to the cells exposed to only the stressor, even so that the values were now not significantly different from the ROS levels in the untreated cells.

### 4.3.2 Glutathione assay

Another way to determine the oxidative stress levels in the proliferating Caco-2 cell is by quantification of the amount of intracellular GSH and GSSG . The results of this assay are depicted in **Figure 16**.

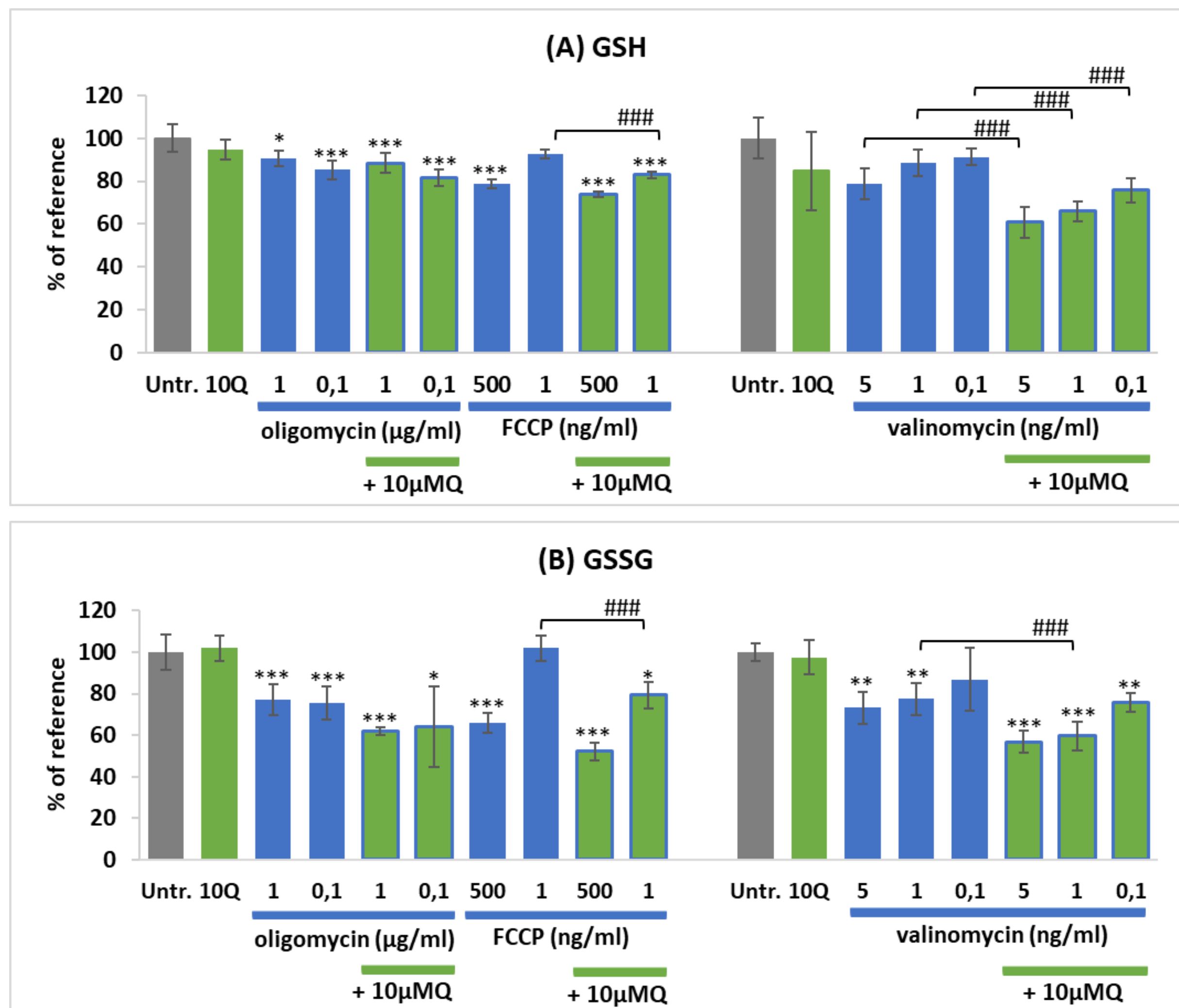


*Figure 16: (A) Mean GSH/SRB relative to untreated cells in proliferating Caco-2 cells. (B) Mean GSSG/SRB relative to untreated cells in proliferating Caco-2 cells. Error bars indicate SD of biological replicates (N=6). *, **, *** denote statistical difference compared to the values for the untreated cells(reference) with p<0.05, p<0.01 and p<0.001, respectively. #, ##, ### denote statistical difference between stressed and non-stressed conditions with p<0.05, p<0.01 and p<0.001, respectively. (Untr. = untreated cells; 10Q = 10µM quercetin)*

The results for the GSH levels as presented in **Figure 16A** showed that quercetin treatment did not significantly alter the GSH levels of the proliferating Caco-2 cells. However, the GSH levels decreased when the cells were stressed with oligomycin (0.1-1 µg/ml), FCCP (500 ng/ml) and valinomycin (5 ng/ml). In addition, co-administration of the stressors with quercetin also resulted in decreased GSH levels, compared to the untreated cells. In case of valinomycin stress, the GSH levels were even lower with quercetin than without quercetin administration.

**Figure 16B** indicate that quercetin exposure did not significantly change the GSSG levels in the proliferating Caco-2 cells. However, the GSSG levels in the proliferating Caco-2 cells decreased when the cells were stressed with oligomycin (0.1-1 µg/ml), FCCP (500 ng/ml) and valinomycin (1- 5 ng/ml). Co-administration of the stressor with quercetin also showed a decrease in the amount of GSSG in the cells compared to the untreated cells. In case of co-administration of quercetin with valinomycin, decreased GSSG levels were observed compared to cells exposed to valinomycin without quercetin, which was a similar trend as observed in the results for the GSH levels under these conditions.

## 4.4 Effect of quercetin on mitochondrial function and respiration of Caco-2 cells

The results above indicated that there may be a link between mitochondrial dysfunction in proliferating Caco-2 cells and the intracellular quercetin content. During the final part of this study, we investigated the influence of quercetin on mitochondrial function (mitochondrial membrane potential, respiration and ATP production), when mitochondrial stress was induced by the mitochondrial stressors oligomycin, FCCP and valinomycin.

### 4.4.1 Mitochondrial membrane potential

The mitochondrial membrane potential in de Caco-2 cells was measured using a spectrofluorometric method using a positively charged stain (TMRE) (**Figure 17**).

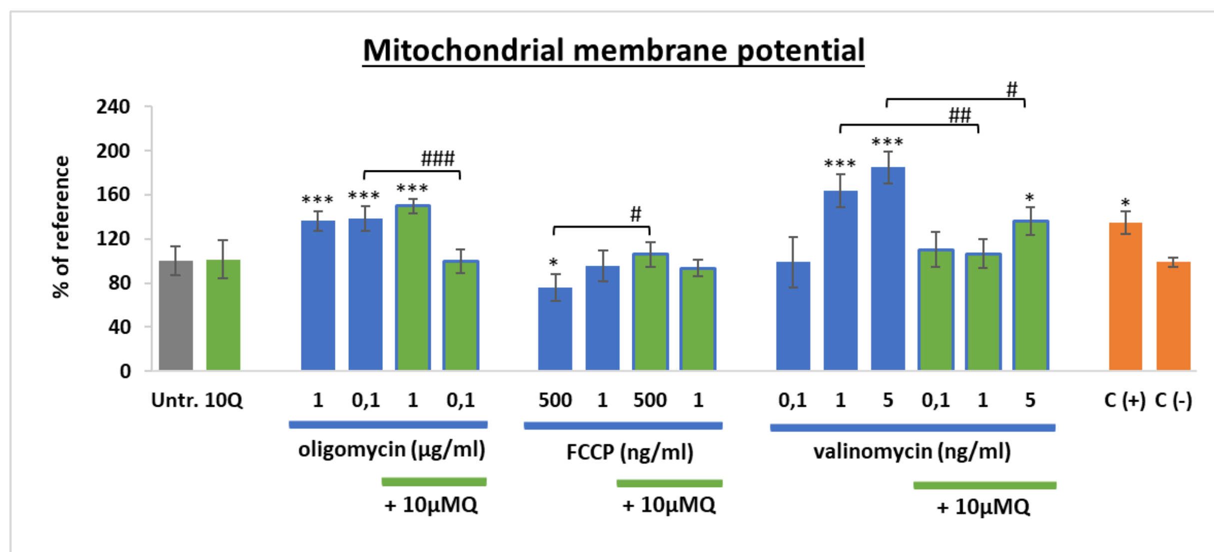


*Figure 17: Mean TMRE/SRB relative to untreated cells in proliferating Caco-2 cells. Error bars indicate SD of biological replicates (N=6). *, **, *** denote statistical difference compared to the values for the untreated cells reference) with p<0.05, p<0.01 and p<0.001, respectively. #, ##, ### denote statistical difference between stressed and non-stressed conditions with p<0.05, p<0.01 and p<0.001, respectively. (Untr. = untreated cells; 10Q = 10µM quercetin) (C(+) = 50 µg/ml oligomycin for 30 minutes as positive control, C(-) = 5 µg/ml FCCP for 10 minutes as negative control)*

First of all, the results shown in figure 17 demonstrate that exposure of the proliferating Caco-2 cells to 10 µM quercetin did not influence the mitochondrial membrane potential. However, exposure to oligomycin and valinomycin, increased the levels of TMRE accumulation. On the other hand, exposure to 500 ng/ml FCCP decreased the levels of TMRE accumulation. Interestingly, co-administration of these stressors with quercetin counteracted these increases or decreases in mitochondrial membrane potential, resulting in a mitochondrial membrane potential which was not significantly different from the untreated cells.

### 4.4.2 Respiration with Seahorse assay

#### 4.4.2.1 Experiment 1: Respiration after long term pretreatment with mitochondrial stressors with or without quercetin in co-administration

The effect on the mitochondrial respiration of proliferating Caco-2 cells after 24h exposure to different mitochondrial stressors (valinomycin, oligomycin and FCCP) with or without 10 µM quercetin in co-administration, was determined using the Seahorse XF Cell Mito Stress Test kit. The basal respiration and respiration after sequential injection of oligomycin, FCCP and rotenone/antimycin A are depicted in **Figure 18-20**.

A first observation was that exposure of the Caco-2 cells to 10 µM quercetin induced a significant decrease in respiration, both basal respiration as respiration after sequential injection of the mitochondrial modulators, compared to the untreated cells.

Pre-treatment of Caco-2 cells with 100 ng/ml oligomycin for 24 hours (**Figure 18**) significantly decreased the basal respiration of the cells, as expected. These results showed that pretreatment of the cells with oligomycin decreased ATP-linked mitochondrial respiration and subsequently mitochondrial ATP production. Note that oligomycin pretreatment of the proliferating Caco-2 cells also caused a reduction in the maximum respiration which is a strong indicator of potential mitochondrial dysfunction. Pretreatment with oligomycin in co-administration of quercetin resulted in lower basal respiration, compared to untreated cells, and no response when mitochondrial modulators were injected.

Similar results were observed when the proliferating Caco-2 cells were exposed to 1 ng/ml FCCP during the 24 hours pretreatment (**Figure 19**): mitochondrial respiration (basal and after injection with modulators) was significantly reduced compared to the untreated cells and no response to the modulators was observed. Interestingly, when the cells were exposed to a pretreatment of 1 ng/ml FCCP in co-administration with 10 µM quercetin, they did react on the injection of FCCP and the maximal mitochondrial respiration increased compared to the mitochondrial respiration of the cells treated for 24 hours with just 1 ng/ml FCCP without quercetin in co-administration.

**Figure 20** showed that exposure of the proliferating Caco-2 cells to 0.1 ng/ml valinomycin for 24 hours with or without 10 µM quercetin did not have a significant effect on the mitochondrial respiration, besides a slight decrease in basal mitochondrial respiration, compared to the untreated cells.

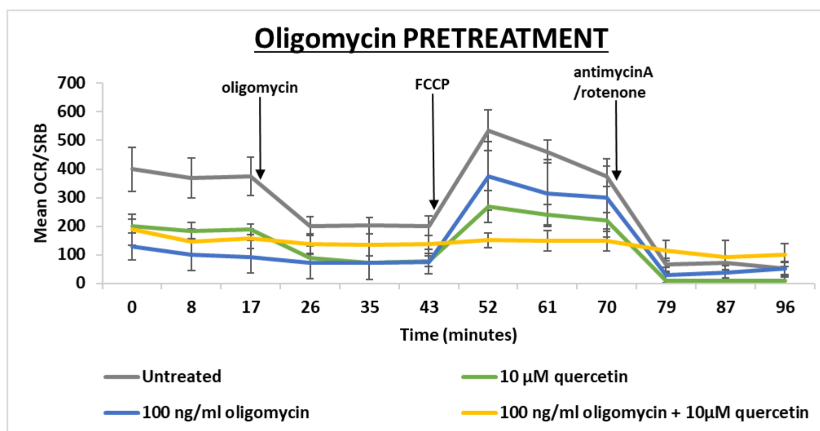


*Figure 18: Mitochondrial respiration after 24h pretreatment with 100 ng/ml oligomycin with or without 10 µM quercetin in co-administration. The mitochondrial respiration of untreated cells and Caco-2 cells treated with only 10 µM quercetin, without any mitochondrial stressor, is also displayed. The results are corrected with the SRB data for cell count and are displayed as the average of the 5 biological replicates at each time point. Error bars indicate SD of biological replicates (N=5).*

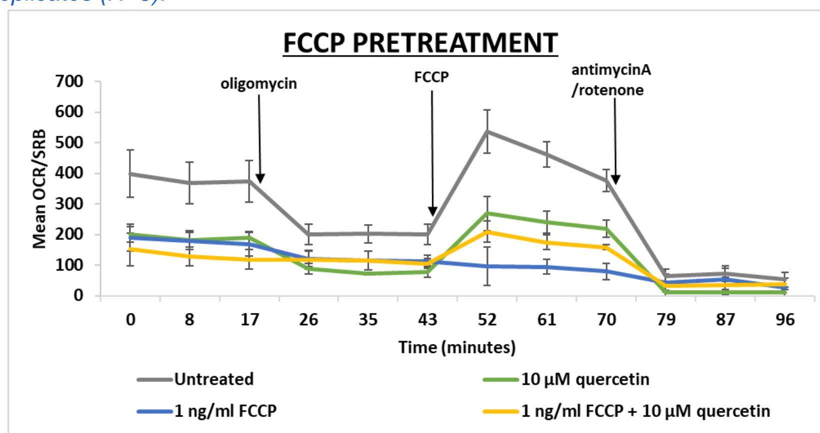


*Figure 19: Mitochondrial respiration after 24h pretreatment with 1 ng/ml FCCP with or without 10 µM quercetin in co-administration. The mitochondrial respiration of untreated cells and Caco-2 cells treated with only 10 µM quercetin, without any mitochondrial stressor, is also displayed. The results are corrected with the SRB data for cell count and are displayed as the average of the 5 biological replicates at each time point. Error bars indicate SD of biological replicates (N=5).*

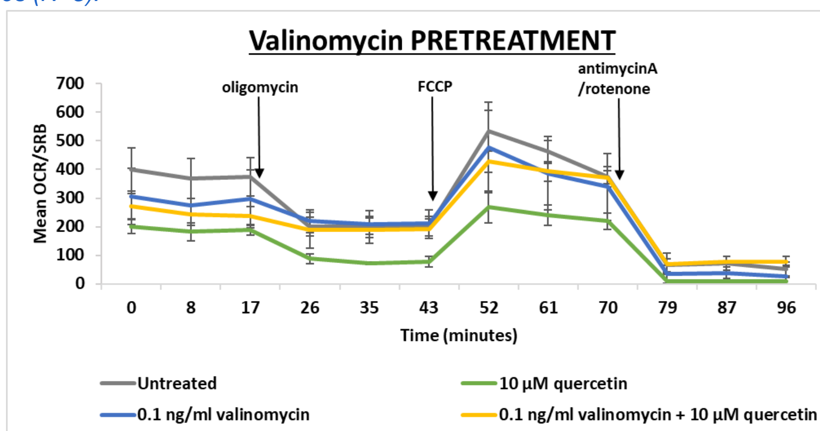


*Figure 20: Mitochondrial respiration after 24h pretreatment with 0.1 ng/ml valinomycin with or without 10 µM quercetin in co-administration. The mitochondrial respiration of untreated cells and Caco-2 cells treated with only 10 µM quercetin, without any mitochondrial stressor, is also displayed. The results are corrected with the SRB data for cell count and are displayed as the average of the 5 biological replicates at each time point. Error bars indicate SD of biological replicates (N=5).*

During the Seahorse assay, not only the oxygen consumption rate (OCR) was measured, but also the extracellular acidification rate (ECAR). These ECAR rates are key indicators of the glycolysis. During the Seahorse assay, the extracellular acidification rate (ECAR) remained constant since mitochondrial modulators were injected and not modulators that effect the glycolysis. **Figure 21** represents the ECAR values measured before the mitochondrial modulators were injected (basal situation).

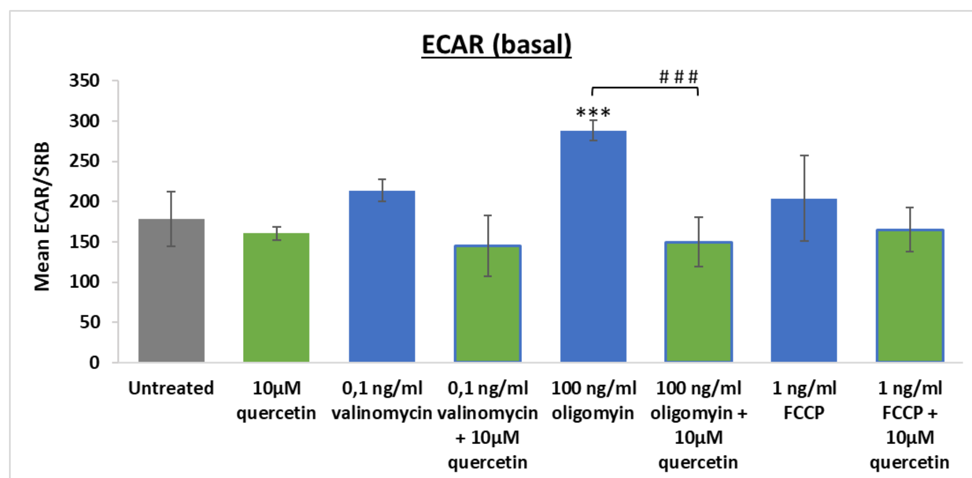


*Figure 21: ECAR values of Caco-2 cells after 24h exposure to mitochondrial stressors with or without 10 µM quercetin in co-administration during the Seahorse assay, before the mitochondrial modulators were added. The ECAR values of untreated cells and cells treated with only 10 µM quercetin, without any mitochondrial stressor, is also displayed. The results were corrected for cell count and are presented as mean values. Error bars indicate SD of biological replicates (N=12). *, **, *** denote statistical difference compared to the values for the untreated cells reference) with p<0.05, p<0.01 and p<0.001, respectively. #, ##, ### denote statistical difference between stressed and non-stressed conditions with p<0.05, p<0.01 and p<0.001, respectively.*

The results depicted in **Figure 21** show that there were significant differences in extracellular acidification between the Caco-2 cells exposed during 24 hours to different treatments. Moreover, addition of 100 ng/ml oligomycin to the proliferating Caco-2 cells for 24 hours significantly increased the extracellular acidification compared to the untreated cells but exposure to a co-administration of oligomycin and quercetin did not induce significant changes in acidification.

#### 4.4.2.2 Experiment 2: Respiration after acute quercetin injection

Figures 18 until 20 showed that quercetin had a clear effect on mitochondrial respiration in proliferating Caco-2 cells. Since some studies suggested the protonophore effect of quercetin, we investigated the effect of directly injecting different concentrations of quercetin (2 µM – 25 µM) on the respiration during a separate Seahorse assay. The respiration curves after sequential injection of oligomycin and different concentrations of quercetin for untreated proliferating Caco-2 cells are depicted in **Figure 22**.

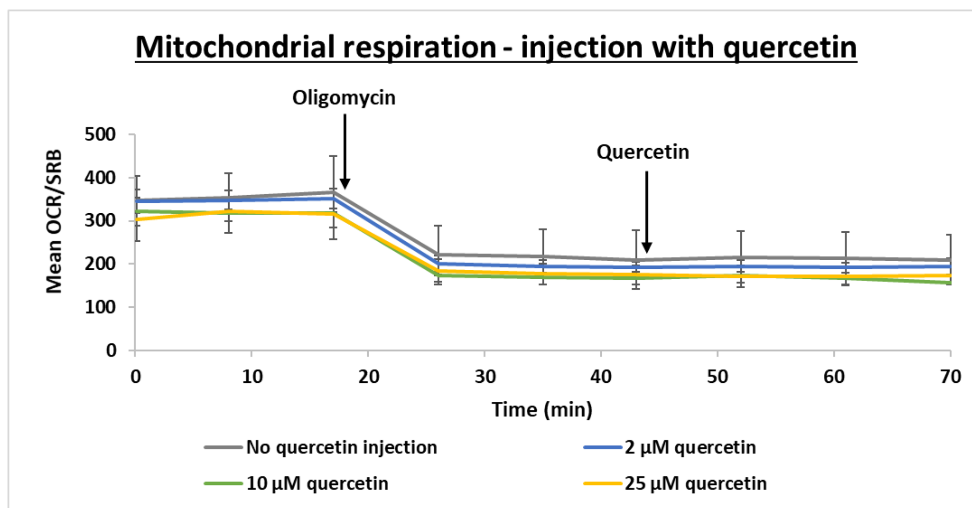


*Figure 22: Mean OCR/SRB (pmol/min) in function of the time. Error bars indicate SD of biological replicates (N=6). At first, 1 µM oligomycin was injected at time = 18 min, after which a second injection with different concentration of quercetin (2 – 25 µM) took place at time = 45 min.*

As shown in **Figure 22**, the basal respiration of the untreated cells corrected for cell count was in line with the basal respiration of the untreated Caco-2 cells as seen in the previous Seahorse assay. Injection of oligomycin decreased the mitochondrial respiration as expected. Moreover, the second injection of different concentrations of quercetin to the untreated proliferating Caco-2 cells had no uncoupling effect on the respiration of the cells.

### 4.4.3 ATP production

To further explore the effects of quercetin on mitochondrial function, the ATP levels were measured in the cells after exposure to the stressor with or without quercetin in co-administration. The results of the assay are depicted in **Figure 23**.

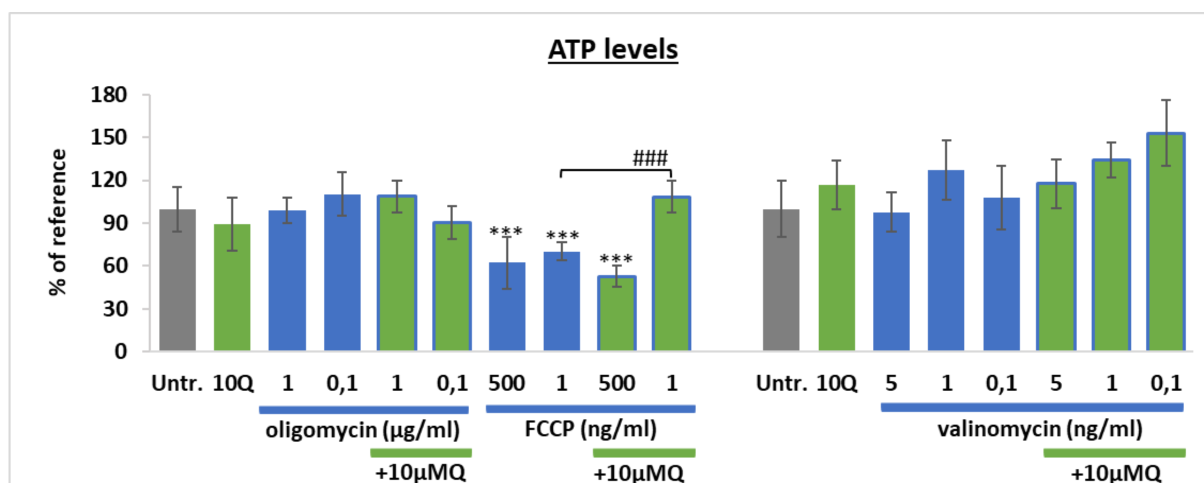


*Figure 23: Mean ATP/SRB relative to untreated cells in proliferating Caco-2 cells. Error bars indicate SD of biological replicates (N=6). *, **, *** denote statistical difference compared to the values for the untreated cells reference) with p<0.05, p<0.01 and p<0.001, respectively. #, ##, ### denote statistical difference between stressed and non-stressed conditions with p<0.05, p<0.01 and p<0.001, respectively. (Untr. = untreated; 10Q = 10 µM quercetin)*

Our results of the ATP assay (**Figure 23**) demonstrated that quercetin did not significantly change the ATP levels in the proliferating Caco-2 cells. In addition, figure 23 shows that the ATP levels in the cells did not significantly change when the cells were stressed with oligomycin or valinomycin compared to the untreated cells. On the other hand, 24 hour exposure to FCCP induced a reduction in ATP levels in the Caco-2 cells as expected. Interestingly, when quercetin was co-administered with 1 ng/ml FCCP with 10 µM quercetin, the ATP depletion was counteracted and the ATP levels were not significantly different from to the ATP levels in the untreated cells.

## 4.5 Overview table results

**Table 3** gives a schematic overview of our results found during the experimental work of this master dissertation.

*Table 3: Schematic overview of the results of the bioassays for each condition. The symbol ↑ means that the values are significantly increased compared to the reference. The symbol ↓ means that the values are significantly decreased compared to the reference and the symbol ≈ states that there is no significant difference compared to the reference. For the results of the ROS levels, GSH levels, GSSG levels, TMRE values and ATP levels the values of the untreated cells were the reference, in case of the intracellular quercetin accumulation the non-stressed cells were the reference.*

| Treatment \ Bio assay | Intracellular quercetin accumulation | ROS levels | GSH levels | GSSG levels | TMRE values | ATP levels |
|---|---|---|---|---|---|---|
| 1 µg/ml oligo. | - | ↑ | ↓ | ↓ | ↑ | ≈ |
| 0.1 µg/ml oligo. | - | ↑ | ↓ | ↓ | ↑ | ≈ |
| 1 µg/ml oligo. + Q. | ↑ | ≈ | ↓ | ↓ | ≈ | ≈ |
| 0.1 µg/ml oligo. + Q. | ↑ | ≈ | ↓ | ↓ | ≈ | ≈ |
| 500 ng/ml FCCP | - | ↑ | ↓ | ↓ | ↓ | ↓ |
| 1 ng/ml FCCP | - | ↑ | ≈ | ≈ | ↓ | ↓ |
| 500 ng/ml FCCP + Q. | ↑ | ↓ | ↓ | ↓ | ≈ | ↓ |
| 1 ng/ml FCCP + Q. | ↑ | ≈ | ↓ | ↓ | ≈ | ≈ |
| 5 ng/ml val. | - | ↑ | ≈ | ↓ | ↑ | ≈ |
| 1 ng/ml val. | - | ↑ | ≈ | ↓ | ↑ | ≈ |
| 0.1 ng/ml val. | - | ≈ | ≈ | ≈ | ≈ | ≈ |
| 5 ng/ml val. + Q. | ≈ | ≈ | ↓ | ↓ | ≈ | ≈ |
| 1 ng/ml val. + Q. | ↑ | ≈ | ↓ | ↓ | ≈ | ≈ |
| 0.1 ng/ml val. + Q. | ≈ | ≈ | ↓ | ↓ | ≈ | ≈ |

# 5 DISCUSSION

In the present master dissertation we described that upon cellular stress, especially mitochondrial stress induced by oligomycin, FCCP and valinomycin, intracellular quercetin accumulation increased significantly in proliferating intestinal cells. In addition, it was demonstrated that the accumulated quercetin could counteract the (i) ROS production induced by oligomycin, FCCP and valinomycin, (ii) ATP depletion induced by FCCP, (iii) changes in the mitochondrial membrane potential induced by all three stressors and (iv) FCCP induced shutdown of the mitochondrial respiration.

## 5.1 Cellular stress induces intracellular quercetin accumulation

First of all, it was observed that the intracellular quercetin accumulation increased when cellular stress was induced in different cell types. The most profound increases in quercetin accumulation were seen in stressed proliferating Caco-2 cells. Exposure of these intestinal cells for 24 hours to the exogenous inflammatory stressor LPS and the mitochondrial stressor valinomycin caused an increase in intracellular quercetin concentration up to 50% of the non-stressed cells. In addition, increases in quercetin accumulation, up to 16 times higher than in non-stressed cells, were reported when mitochondrial dysfunction was induced using the mitochondrial stressors oligomycin or FCCP. However, the effect of cellular stress on the intracellular accumulation of quercetin was not only limited to intestinal Caco-2 cells. We also reported significant increases in proliferating EA.hy926 cells when the cells were stressed with oligomycin, but not with FCCP, and in quiescent EA.hy926 cells when the cells where stressed with FCCP, but not with oligomycin. Our results of the accumulation assay give rise to the idea that different types of cellular stress may increase the intracellular quercetin accumulation since multiple stressors show the same effect but the extent of the increased accumulation could be stressor and cell type specific. The fact that the most noticeable increases in intracellular quercetin accumulation were seen in cells stressed by specific mitochondrial stressors and LPS, which can also indirectly induce mitochondrial dysfunction, suggests that mitochondrial (dys)function may play an important role in the mechanism that induces the observed effects.

As described in section 1.2.2, mitochondrial dysfunction is associated with the inflammation pathway. However, upon pro-inflammatory TNF-α induced stress, which may also induce mitochondrial dysfunction (Chen et al., 2010; Mariappan et al., 2007) a significant decrease in quercetin accumulation was observed. Although previous *in vitro* studies have shown that the used TNF-α concentrations are not cytotoxic to Caco-2 cells, it should be noted that in *vivo* studies report that the plasma concentrations of TNF-α in populations that are at increased risk of developing chronic diseases (obese, elderly etc.), rarely exceeds 14 pg/ml (Bruunsgaard et al., 1999; Zahorska-Markiewicz et al., 2000). It can be suggested that in addition to the type of stressor and the cell type, the concentration of the stressor and exposure time also plays an important role in the mechanism behind the increase in quercetin accumulation upon cellular stress.

In general, the accumulation of flavonoids in the cell is dependent of the influx and efflux of flavonoids by passive diffusion and membrane transport proteins, as discussed in the literature study of this work. Based on this, we suspected that an increase in quercetin accumulation is associated with (i) an increase in passive diffusion due to an extracellular increase in quercetin concentration, (ii) an increase in quercetin influx by influx transporters, (iii) a decrease in quercetin efflux by ABC transporters or (iv) a combination of the previous. We performed a range of high throughput assays in order to better understand how cellular stress, especially mitochondrial stress, may influence these processes and subsequently the accumulation of quercetin.

Although the mechanism of action of the mitochondrial stressors that increased quercetin accumulation is different, they all (i) altered the mitochondrial membrane potential, (ii) increased ROS levels and (iii) decreased basal respiration and subsequently mitochondrial ATP production. On the other hand, results showed a significant decrease in total ATP levels in FCCP treated Caco-2 cells but no changes in Caco-2 cells treated with oligomycin and valinomycin. Literature studies reported that in many cases of mitochondrial stress, a shift is made towards more cytoplasmic glycolysis to keep the ATP production on point which could explain why ATP levels remain constant in oligomycin and valinomycin treated cells (Garedew et al., 2010; Liu et al., 2006; Stevens et al., 2018; S.-B. Wu & Wei, 2012). These studies suggested that the metabolic shift from respiration to glycolysis is induced through the activation of AMP-protein kinase (AMPK) by oxidative stress (S.-B. Wu & Wei, 2012) or changes in MMP (Garedew et al., 2010; Stevens et al., 2018). Since our results showed that each of the mitochondrial stressors increased the oxidative stress levels in the cell and changed the MMP, it is plausible that the shift towards more glycolysis is induced through this pathway. Since the sugar content remained constant during the assays, we suspect that the amount of sugar in the medium did not significantly influence the glycolysis and subsequently our results, but additional research is recommended to confirm this.

A known attribute of increased glycolysis is an increase in extracellular acidification by lactate. Our results showed that the extracellular acidification rate was significantly increased when Caco-2 cells were exposed for 24 hours to oligomycin which further reinforces our hypothesis that mitochondrial dysfunction caused a shift to more glycolysis. Interestingly, studies have shown that OATP transporter activity increases with decreasing extracellular pH which leads us to the hypothesis that the increased glycolysis, induced by mitochondrial dysfunction, may increase the extracellular acidification and in turn, increase the accumulation of quercetin by OATP transporters. It is proposed that a decrease in extracellular pH increases the substrate affinity because of the protonation of a conserved histidine residue at the extracellular end of transmembrane domain 3 of OATP transporters (Leuthold et al., 2009). Moreover, Leuthold et al. (2009) already suggested that quercetin may be acting as a high-affinity substrate of OATP in order to enter the cell at low pH values in intestinal Caco-2 cells.

Furthermore, the acidic conditions could also induce the activity of certain enzymes such as β-glucuronidase, this way increasing the conversion of quercetin glucuronides into the more bioactive and more easily transported quercetin aglycone.

**Figure 24** represents a mechanism proposed by Kawabata et al. (2015) for the accumulation of quercetin in macrophages. Our results show that the increase of quercetin accumulation in proliferating Caco-2 cells during mitochondrial dysfunction could be similar to this mechanism.

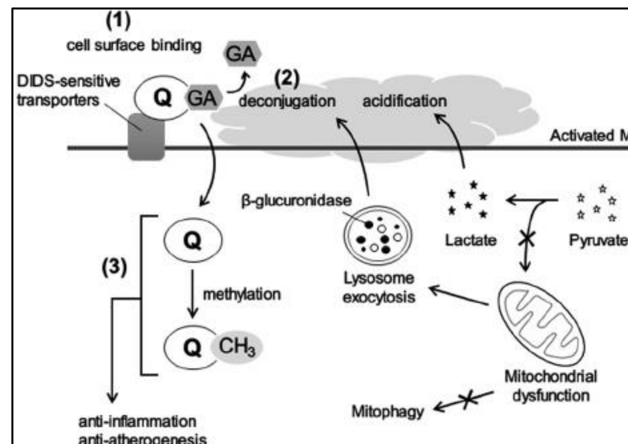


*Figure 24: Proposed scheme for the accumulation and deconjugation of quercetin glucuronide in macrophages by Kawabata et al. (2015). (1) the quercetin glucuronide (Q3GA) binds to the cell surface protein. (2) The conjugate is deconjugated into the aglycone (Q) by β-glucuronidase. The deconjugation activity of β-glucuronidase is promoted by acidification via the secretion of lactate induced by mitochondrial dysfunction. (3) The aglycone (Q) passes through cell membranes, is partially methylated (Q-CH3) in the cells by their metabolism, and then exerts various functions such as anti-inflammatory and anti-atherosclerotic activities. DIDS = 4, 4'-diisothiocyanatostilbene-2,2'-disulfonic acid; GA, glucuronic acid; Q, quercetin.*

Moreover, it is plausible that the decrease in ATP levels caused by FCCP, decreased the efflux of quercetin by ABC transporters, this way contributing to a significant increase in quercetin accumulation compared to the unstressed cell.

In addition to the extracellular pH and the amount of ATP available, the activity of transporters can also be directly influenced by the stressors used to induce cellular stress. For example, it has been reported that the activity of some OATP transporters is inhibited by changes in the plasma membrane potential induced by valinomycin (Martinez-Becerra et al., 2011). Moreover, valinomycin is also a known inhibitor of the ABC transporter P-glycoprotein (Chabane, Ahmad, Peluso, Muller, & Ubeaud-Séquier, 2009), which may explain the increased quercetin accumulation upon valinomycin induced stress. In addition, the proton ionophore FCCP can uncouple a proton gradient over the plasma membrane therefore decreasing the activity of the OATP2B1 transporter in Caco-2 cells (Varma et al., 2011), which can result in less quercetin efflux. Moreover, it has also been reported that quercetin may follow the FCCP-driven proton influx, resulting in higher quercetin uptake (Glaeser et al., 2014). Nevertheless, it is important to note that intracellular quercetin accumulation was also increased significantly when cells are stressed with oligomycin. Since oligomycin has no direct effect on the plasma membrane potential or transporters, we suspect that changes in plasma membrane potential directly induced by FCCP and valinomycin may influence the ability of quercetin to penetrate intracellularly under stressful conditions but is not the main cause of this effect.

In addition, not only the mitochondrial stressors can influence the transporters but also the inflammatory stressors TNF-α and LPS. For example, decreased Pgp and Mrp2 levels have been associated with LPS-treated rats, which may partially explain the observed increase in quercetin accumulation. Exposure to TNF-α (1-100 ng/ml), on the other hand, could potentially down-regulate mRNA levels of influx transporters such as OATP1B1, OATP1B3 and OATP2B1 and up-regulate the expression of efflux pumps such as BCRP and MRP (Le Vee, Lecureur, Stieger, & Fardel, 2009), suggesting an increased quercetin efflux upon TNF-α stress.

Since many studies point out the difference in cellular stress response between different cell lines, it is not unexpected that our results showed significant differences in quercetin accumulation between them. Moreover, the difference in membrane transport protein composition between different cell lines, including influx and efflux transporters, may contribute to this observation (Holla et al., 2008). Since we suspect that the increase in quercetin accumulation is connected with the mitochondria in the cell, it is interesting to keep in mind the possibility that the relative amount of mitochondria could also influence the extent of the effect.

## 5.2 Protective response towards the induced cellular stress

Together with the increase in quercetin accumulation upon cellular stress, a protective response towards the induced cellular stress was observed.

### 5.2.1 Impact of quercetin on oxidative stress levels

First, the increase in quercetin accumulation induced by cellular stress caused an increased bioactivity of quercetin against oxidative stress levels in the Caco-2 cells. Since the amount of reactive oxygen species in the stressed cells exposed to quercetin was significantly lower than the amount of ROS in the unstressed cell, it is very unlikely that the decrease is due to the direct antioxidant capacity of quercetin alone. In addition, an increasing amount of review papers report that the direct contribution of quercetin to the redox status is likely to be extremely low compared to other more stable endogenous oxidizable substrates (Hollman, 2014). In addition, researchers are now focused on more complex effects of quercetin on molecular targets and enzymes in order to modulate the cellular redox state, including their capacity to increase endogenous antioxidant defenses. For example, it is reported that quercetin induces Nrf2 nuclear translocation and DNA binding activity via activation of p38 which results in gene transcription and protein expression of antioxidant enzymes such as HO-1 and GCL.

In addition, the antioxidant effect of the flavonoids to reduce ROS could be similar to the mechanism proposed in **Figure 25**. Note that low to moderate elevations in ROS levels act as signaling molecules and also induce Nrf2 activation and in turn expression of these antioxidant enzymes to maintain physiological functions. In this context, we have to keep in mind that there is a tight balance between increased ROS levels that increase the endogenous antioxidant system and oxidative stress which may lead to augmented release of pro-inflammatory cytokines (López-Armada et al., 2013).

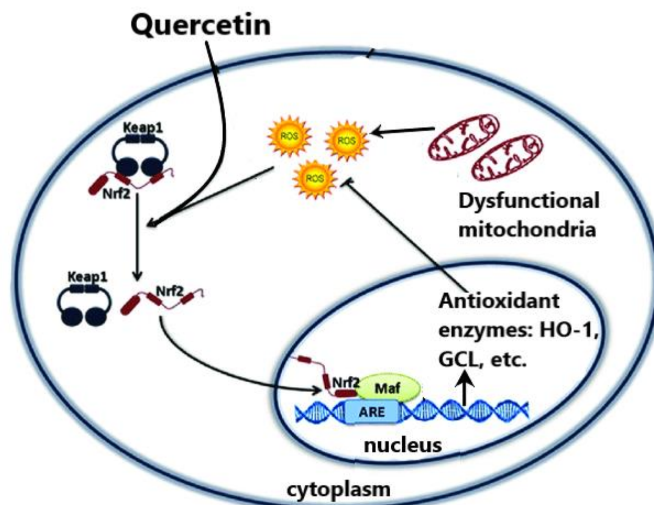


*Figure 25: Hypothetical figure summarizing the antioxidant effects of quercetin in stress induced cells. Quercetin and/or ROS induces Nrf2 nuclear translocation which results in gene transcription and protein expression of the antioxidant enzymes, HO-1, NQO1, and GCL, which in turn inhibit reduced mitochondrial stressor induced formation of oxidants (ROS) (Khadrawy et al., 2019).*

One of those endogenous antioxidant defenses induced by Nrf2 is the production of the antioxidant enzyme glutathione by upregulation of the formation of γ-glutamylcysteine synthetase, which is the rate limiting step of the glutathione synthesis. The induction of this enzyme by quercetin may give the cells an increased ability to rapidly synthesize GSH during an oxidant challenge. Studies showed that quercetin is the most potent flavonoid when it comes to induction of γ-glutamylcysteine synthesis which emphasizes the specificity of γGCSh induction (C. Li, Zhang, Choi, & Frei, 2016; Moskaug et al., 2005).

However, the complexity of this system has been displayed in our results of our glutathione assay. The GSH levels in all stressed cells with or without quercetin in co-administration showed no or very small significant decreases compared to the non-stressed cells. Unexpectedly, the GSSG levels are also decreased when the cells were stressed with the mitochondrial stressors with or without quercetin. Even if we take into account the fact that GSSG can be reduced back to GSH by glutathione reductase, using NADPH as an electron donor, this does still not explain the decrease in both GSH and GSSG levels which gives rise to the idea that the changes in glutathione levels are not only a result of redox transformations but also influenced by the synthesis and export of glutathione and glutathione conjugates. For example, studies report that changes in the glutathione formation can be induced by Nrf2 activation by increased ROS levels and/or quercetin. For this reason, we expected an increase in GSH levels, which was not observed. On the other hand, the decrease in GSH and GSSG levels may be induced by increased efflux of these components out of the cell. For example, studies showed that quercetin induces significant increases in MRP1 protein levels in Caco-2 cells which is an ABC transporter known to be involved in cross membrane cellular export of glutathione and glutathione conjugates. Since quercetin is only a substrate of MRP2 and not a substrate of MRP1 in Caco-2 cells, quercetin levels are not affected by this increase in MRP1 protein levels (Chabane et al., 2009). In addition, studies show in general an increase in OATP transporter expression during stressed conditions which may have a hand in the efflux of glutathione since OATP transporters couple the uptake of organic

anions to the efflux of bicarbonate or glutathione (Leuthold et al., 2009; L. Li et al., 2000; Petrovic et al., 2007). Furthermore, it must be noted that quercetin is oxidized to a quinone when serving as an antioxidant after which it can react with glutathione and form quercetin glutathione conjugates. Interestingly, studies reported that in most cases flavonoid-glutathione conjugates retain the redox and electrophilic properties of the parent flavonoid but the active transport of these conjugates out of the cell into the bloodstream limits their potential reactivity in the cells (Monks & Lau, 1997, 1998). In addition, the OPT stain used in the glutathione assay cannot bind to glutathione when it has formed conjugates with quercetin, which could also be a possible explanation for the reduction in GSH levels.

In conclusion, **Figure 26** gives an hypothetical overview of a possible mechanism by which quercetin influences glutathione levels based on a study by C. Li, Zhang, Choi, et al. (2016). However, based on our results of the glutathione level there is too much uncertainty to make conclusive results.

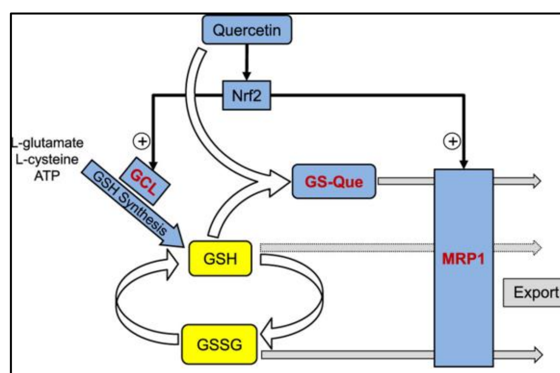


*Figure 26: possible mechanism by which quercetin influences glutathione levels based on a study by C. Li, Zhang, Choi, et al. (2016). Quercetin activates Nrf2 which in turn upregulates the formation of γ-glutamylcysteine synthetase (GCL) and the ABC transporter MRP1. The increase in GCL leads to the synthesis of GSH. GSH coverts into GSSG depending on the oxidative stress level in the cell. In addition, quercetin may form conjugates with glutathione that are transported out of the cell, together with GSH and GSSH by MRP1.*

More recent papers suggest that the antioxidant capacity of quercetin is an indirect result of their pro-oxidant capacity by dissipating the mitochondrial membrane potential via a protonophore mechanism (Stevens et al., 2018). According to their hypothesis, this pro-oxidant effect will deplete GSH levels in the cell and trigger an adaptive stress response leading to GSH synthesis to protect cells from pre-existing ROS. However, our results showed that 24h exposure of proliferating Caco-2 cells to 10 µM quercetin did not change the mitochondrial membrane potential and did not increase GSH levels in the cells compared to untreated cells. Moreover, the ROS levels in Caco-2 cells exposed to quercetin were also not significantly different from the ROS levels in untreated cells. In addition, acute quercetin exposure after injection of different concentrations of quercetin (2-25µM) showed no changes in the respiration curve and hence no uncoupling effect as proposed in those studies (Stevens et al., 2018). In conclusion, our results show no proof for this hypothesis. Nevertheless, none of the studies reviewed by Stevens et al. (2018), that reported the depolarizing effect of polyphenols on the mitochondrial membrane, worked with intestinal Caco-2 cells so the type of cell could play an important role.

### 5.2.2 Impact of quercetin on mitochondrial (dys)funtion

Besides the protective effect of increased quercetin accumulation against oxidative stress levels, our findings also showed a beneficial effect on mitochondrial function. First of all, the increased quercetin accumulation counteracted the significant changes in mitochondrial membrane potential induced by all three mitochondrial stressors. Note that our results showed that oligomycin stress resulted in hyperpolarization of the mitochondrial membrane, while exposure to FCCP decreased the mitochondrial membrane potential. These results are in line with literature since oligomycin blocks ATPase and FCCP works as a proton carrier across the membrane (Zorova et al., 2018). However, our results showed an increase in mitochondrial membrane potential when the cells were exposed to valinomycin which is unexpected since it is described that valinomycin lowers the MMP due to the uptake of $K^+$ as positively charged ionophore complex (Teplova et al., 2006). However, this outcome could be the result of the set-up of the experiment and the choice of valinomycin concentrations (Teplova et al., 2006).

The observation that quercetin nullified the changes in mitochondrial membrane potential induced by the stressors suggest that quercetin may exert a direct effect on the mitochondria. Since studies have shown that quercetin can accumulate in the plasma membrane of Caco-2 cells and hence influence transporters located at these membranes (Gonzales et al., 2015), it is not surprising that quercetin may also be located in the mitochondrial membrane. A few studies already suggested that quercetin interacts directly with mitochondrial membranes causing alterations in their fluidity, affecting the function of proteins located in the membranes and interacting with components of the mitochondrial electron transport chain, this way inhibiting and/or uncoupling mitochondrial respiration (Carrasco-Pozo et al., 2012; Dorta et al., 2005; Stevens et al., 2018). In addition, the effect of quercetin on mitochondrial respiration is also represented in our results of the Seahorse assay which showed a decrease in basal mitochondrial respiration when the proliferating Caco-2 cells were exposed to 10 µM quercetin whether or not in co-administration with one of the stressors. Our results are in line with studies that suggested that quercetin could inhibit ATPase due to the structural similarity between quercetin and oligomycin and may inhibit complex I after binding to the ubiquinone site of complex I (Carrasco-Pozo et al., 2012; Stevens et al., 2018). The inhibition of the electron transport chain by quercetin could also have a hand in the decreased ROS levels in the cells since these complexes are a big contributor to mitochondrial ROS production.

Besides the general decrease in mitochondrial respiration after exposure to quercetin, our results also showed that exposure of FCCP induced stressed Caco-2 cells to quercetin at least partly counteracted the complete respiratory shutdown induced by FCCP since the respiration curve showed a reaction to injection of FCCP. This effect was also seen in our results of the ATP assay and MMP assay which showed that exposure to quercetin nullified the significant decrease in ATP levels and mitochondrial membrane potential induced by FCCP.

In addition, the respiration curve for proliferating Caco-2 cells treated with 0.1 ng/ml valinomycin was not significantly different from the respiration curve of Caco-2 cells exposed to 0.1 ng/ml valinomycin in co-administration with quercetin. This is not unexpected since our results showed that the mitochondrial

membrane potential, ATP levels, ECAR levels and even ROS levels are not significantly different between these two conditions.

However, our results also showed some contradictory findings regarding Caco-2 cells exposed to oligomycin with quercetin in co-administration. Co-administration of oligomycin with quercetin significantly reduced the ECAR values compared to the values after treatment with only the stressor, back to the same level as the untreated cells. These results could imply that the accumulated quercetin reduced the glycolysis or prevented the metabolic shift towards increased glycolytic metabolism since studies reported already the inhibitory effect of quercetin on the glycolysis. Moreover, our results of the MMP assay showed no significant changes in MMP, ATP levels and ROS content compared to the untreated cells, hence demonstrating that co-administration with quercetin has a positive effect on mitochondrial function in the cell. However, our results of the Seahorse assay showed that exposure of Caco-2 cells to oligomycin in co-treatment with quercetin significantly decreased mitochondrial respiration to the point that cells did not respond to injection of the mitochondrial modulators as seen when cells were treated with FCCP. From these results it looked like mitochondrial function did not improve at all by increased quercetin accumulation.

The protective effect of quercetin on mitochondria in cultured cells and *in vivo* during non-stressed conditions has been reviewed by de Oliveira et al. (2016). They linked the protective effect of quercetin on mitochondrial function to the capacity of quercetin to activate Nrf2 although they reported that a causative link between Nrf2 activation and alterations in mitochondria related redox and/or functional parameters has not been found yet. However, based on our results it is very plausible that quercetin alters mitochondrial function by direct interactions with mitochondrial membranes and/or components involved in mitochondrial processes. However, additional research is necessary to better understand the location of quercetin in mitochondria, its interaction with different components of the mitochondrial pathways and the role of cellular stress on both localization and these interactions.

In conclusion, **Figure 27** shows an overview of a potential mechanism behind the increased intracellular quercetin accumulation upon stress. This figure is hypothetical and is created based on literature and own observed results.

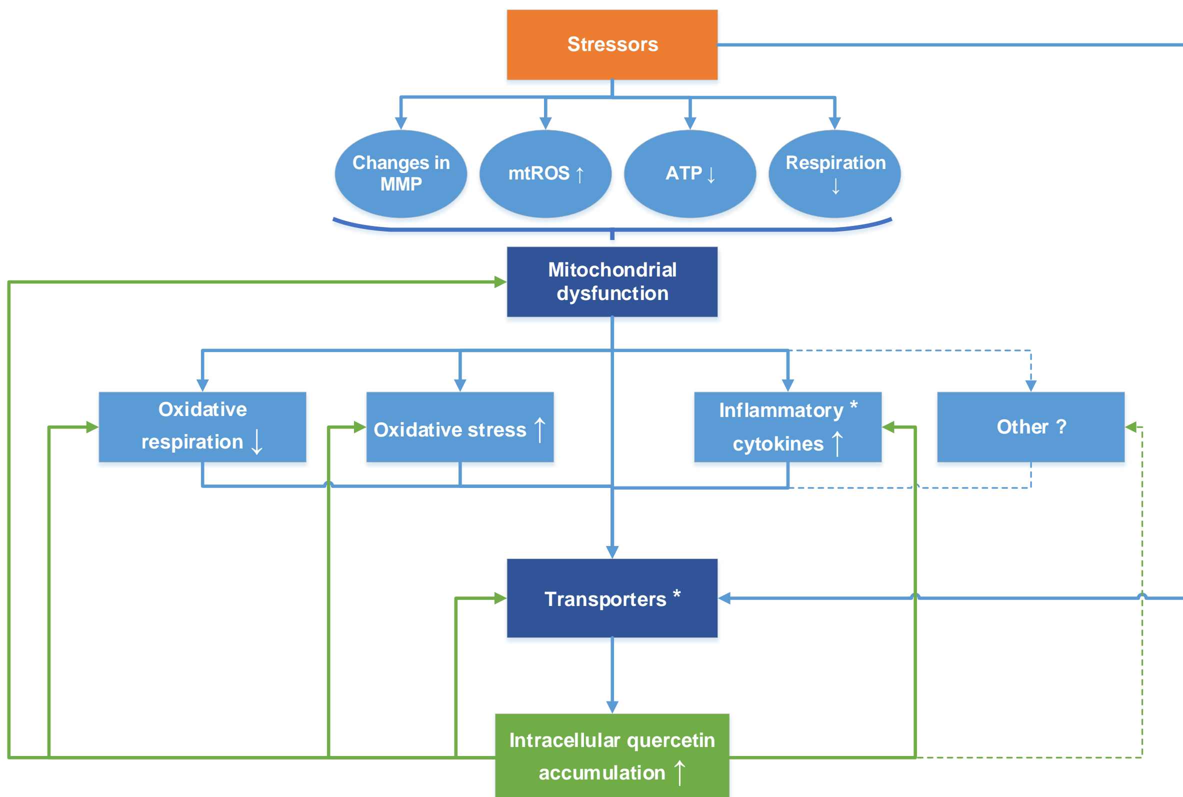


*Figure 27: overview of a potential mechanism behind the increased intracellular quercetin accumulation upon stress. Exposure of proliferating Caco-2 cells induces mitochondrial dysfunction in the cell. In turn, mitochondrial dysfunction may induce a cellular stress response that leads to a decrease in oxidative respiration and an increase in oxidative stress levels and inflammatory cytokines. In turn, this cellular stress response may influence the activity of transporters located at the plasma membrane which may result in the increased quercetin accumulation in the cell. Additionally, the accumulated quercetin may improve mitochondrial function, oxidative stress levels and other cellular functions. (* denote that this parameter is not measured in this study).*

# 6 CONCLUSION

In this study, the effect of cellular stress caused by a range of mitochondrial and inflammatory stressors on the intracellular accumulation of quercetin in different cell lines was investigated. Our results indicate that cellular stress increases the ability of quercetin to penetrate intracellularly. Furthermore, the increase in quercetin accumulation occurred in conjunction with a reduction of stress induced ROS production and a recovery of mitochondrial function in intestinal Caco-2 cells.

In addition, our findings suggested that cellular stress could potentially induce a cellular stress response mechanism that will influence the activity of transporters who control the influx and efflux of quercetin. The fact that the most significant increases in quercetin accumulation are observed when cellular stress is induced by mitochondrial stressors, suggests that mitochondrial dysfunction plays a central role in this stress response mechanism.

On the other hand, we can also conclude, based on our observations, that the extent of the cellular stress response and subsequently the increase in quercetin accumulation is highly dependent on the type of stressor that is used to induce mitochondrial dysfunction, the concentration of this stressor and the cell type. In conclusion, extended research is necessary to further investigate the pathways leading to the increase in quercetin accumulation and recovery of mitochondrial function.

Finally, this study indicates the importance of the stratification of the population, based on cellular stress or health status, when studying quercetin bio-availability or bio-activity. For example, it can be suggested that a more consistent and significant impact of quercetin on human health can be revealed in clinical trials, when subpopulations with cellular stress are targeted. Based on our preliminary *in vitro* data, it may be hypothesized that subpopulations which encounter cellular stress, associated with chronic diseases, may experience more beneficial health effects from quercetin consumption compared to healthy subpopulations.

# 7 FUTURE PERSPECTIVES

In order to better understand whether and how quercetin elicits mitochondrial protection in cultured cells we are interested if quercetin is able to reach and even accumulate in the mitochondria. Studies already reported accumulation of quercetin in isolated mitochondria of other cell lines thereby implying that mitochondria represent a reservoir of biologically active quercetin (Fiorani et al., 2010). Therefore, it would be interesting to investigate if quercetin accumulates in the mitochondria of intestinal cells, and what the effect of cellular stress is on mitochondria and the localization of quercetin.

Additionally, in this study we focused on one cell type, three very specific stressors and the flavonoid quercetin since this is the most abundant flavonoid in the human diet. The question now is whether our results also apply to other flavonoids. We already suggested that the cellular stress response induced by mitochondrial dysfunction has an effect on the activity of the transporters located in the plasma membrane. Therefore, we could suspect that the effect of cellular stress on the accumulation of other flavonoids will likely depend on their affinity with these transporters. In turn, we suspect that the bioactivity of the accumulated flavonoids in the cell will also depend on the interaction of the flavonoid with the different molecular targets and enzymes. In conclusion, we expect that the effect of cellular stress on the bioavailability and bioactivity of flavonoids can be highly dependent on the flavonoid itself, which has been demonstrated by our research group before. More research is therefore needed to investigate this further. Therefore, long term, it would be interesting to extend the study to other flavonoids, cell lines and stressors. In addition, since we focus now on monocultures it would be interesting to use more complex cell models to investigate also the influence of cross-talk between different cell types.

Finally, during this study we always assumed that the stressors did not directly interact with quercetin when we added both the stressor and quercetin in co-administration to the cells. However, it would be interesting to rule-out a direct interaction of the stressors with quercetin as an explanation for the protective effect of quercetin against mitochondrial dysfunction. In this case, quercetin profiles could be examined by reverse HPLC after incubation in presence or absence of the stressor. However, it is very unlikely that quercetin has a direct neutralizing effect on all three different stressors since this scenario would not explain the increase in intracellular quercetin accumulation.

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# APPENDIX

## Appendix 1: Recent (2013-2018) studies on the influence of different flavonoids on TNF-α induced stress in various cell lines

*Table 3- Recent (2013-2018) studies on the influence of different flavonoids on TNF-α induced stress in various cell lines*

| Cell line | Flavonoid (concentration) | Stress factor (concentration) | Methods used in the study | Result of addition flavonoid to stressed cell line compared to stressed cell without the flavonoid | Reference |
|---|---|---|---|---|---|
| CaCo-2 | Daidzein + derivatives of daidzein (0,01-100µM) | TNF-α (2 ng/ml) | - WST-1 assay<br>- ELISA<br>- Western blot | - IL-8 production ↓<br>- Phosphorylation of JNK ↓<br>- Derivatives have better anti-inflammatory activities then parent daidzein. | Peng et al. (2017) |
| Caco2/ HUVEC Coculture | Cyanidin-3-O-Glucoside (C3G) (20 µM) | TNF-α (50 ng/ml) | - Leukocyte adhesion assay<br>- Western blot<br>- Quantitative RT-PCR | - Nuclear translocation NF-κB in Caco-2↓<br>- TNF-α and IL-8 gene expression in Caco-2 ↓<br>- E-selectin and VCAM-1 mRNA in HUVEC ↓<br>- Leukocyte adhesion ↓<br>- NF-κB levels in HUVEC ↓ | Ferrari et al. (2017) |
| Caco-2/TC7 | Biochanin A + Prunetin (50 µM) | TNF-α (100 ng/ml) | - TEER<br>- Luciferase reporter gene assay | - Barrier disruption ↓<br>- NF-κB activation ↓ | Piegholdt et al. (2014) |
| Differentiated CaCo-2 | O-glucosides of cyanidin, delphinidin, (0.25µM-1µM) | TNF-α (5ng/ml) | - TEER<br>- FITC-dextran paracellular transport<br>- Western blot | - TEER ↓<br>- FITC-dextran permeability ↓<br>- Effect was not seen for O-glucosides of malvidin, peonidin and petunidin | Cremonini et al. (2017) |
| Differentiated Caco-2 | Cyanidin-3-glucoside (20-40µM) | TNF-α (50ng/ml) | - Western blot<br>- RT-PCR<br>- GSH content assay | - Nuclear translocation of p65 ↓<br>- IKK phosphorylation ↓<br>- IkBa degradation ↓<br>- Expression IL-6 and COX-2 ↓<br>- GSH depletion ↓ | Ferrari et al. (2016) |
| HEK293 | Kaempferol (5-40 µM) | TNF-α (15-100 ng/ml) | - NF-κB reporter assay | - Dose dependent inhibition of NF-κB activity | Kadioglu et al. (2015) |

| HT-29 intestinal cells | Cyanidin-3-glucoside + resveratrol | IL-1α (10 ng/ml) + TNF-α (20 ng/ml) + IFN-γ (60 ng/ml) | - ELISA<br>- Quantitative RT-PCR<br>- GSH and GSSG content assay<br>- ROS assay via DCFH2-DA | - Nrf2 activation ↑<br>- GSH/GSSG ratio ↑<br>- ROS levels ↓ | Serra et al. (2016) |
|---|---|---|---|---|---|
| HT-29/B6 | Quercetin (200µM) | TNF-α (10ng/ml) | - TEER | - Total resistance in HT-29/B6 cell monolayers ↑ | Amasheh et al. (2012) |
| EA.hy926 | Luteolin (0.5µM, 2µM, 20µM) | TNF-α (10 ng/ml) | - Leukocyte adhesion assay<br>- Western blot<br>- RT-PCR | - Binding monocytes to cells ↓<br>- Expression of chemokines ↓<br>- Expression ICAM-1 and VCAM-1 ↓<br>- NF-κB transcriptional activity ↓<br>- IκBα degradation ↓<br>- NF-κB p65 nuclear translocation ↓ | Jia et al. (2015) |
| HUVEC | Cyanidin-3-Oglucoside (C3G) (20 - 40 µM) | TNF-α (20 ng/ml) | - Quantitative RT-PCR<br>- Superoxide dismutase assay<br>- GSH and GSSG content assay<br>- Western blot | - Oxidative stress ↓<br>- Activation Nrf2 pathway ↑<br>- Involvement of MAPKs (ERK1/2) in C3G induction of Nrf2 pathway.<br>- NF-κB p65 nuclear translocation ↓ | Speciale et al. (2016) |
| HUVEC + U937 cells | Genistein (0.1 - 10 µM) | TNF-α (2 ng/ml) | - Leukocyte adhesion assay using calcein-AM labeled U937 cells<br>- ELISA<br>- Intracellular cAMP assay<br>- EPR spectroscopy + DEPMPO/DMPO-spin trapping technique<br>- Glutathione (GSH) and antioxidant enzymes assays | - Adhesion monocytes to HUVEC ↓<br>- Production adhesion molecules and chemokines ↓<br>- Genistein at concentrations < 10 µM had no significant free radical scavenging activity<br>- Genistein has no effect on induction of endogenous antioxidants enzymes<br>- The anti-inflammatory effect of genistein may be mediated at least partially through the PKA-mediated mechanism | Jia et al. (2013) |
| HUVEC + U937 cells | Naringenin and Hesperetin metabolites (0,5 -10 µM) | TNF-α (0,1 ng/ml) | - Monocyte adhesion assay<br>- Quantitative RT-PCR<br>- Western blot | - Monocyte adhesion at endothelial cells ↓<br>- Flavonoids affect the expression of related genes | Chanet et al. (2013) |
| HUVEC + THP-1 | Cyanidin, malvidin, delphinidin (0,1-10 µg/mL) | TNF-α (100 ng/ml) | - MTT assay<br>- Monocyte adhesion assay with CellTracker Green CMFDA | - Monocytes adhesion to endothelial cells ↓ | Del Bo' et al. (2016) |

## Appendix 2: Recent (2013-2018) studies on the influence of different flavonoids on LPS induced stress in various cell lines

*Table 4- Recent (2013-2018) studies on the influence of different flavonoids on LPS induced stress in various cell lines*

| Cell line | Flavonoid (concentration) | Stress factor (concentration) | Methods used in the study | Result of addition flavonoid to stressed cell line compared to stressed cell without the flavonoid | Reference |
|---|---|---|---|---|---|
| Caco-2 | Resveratrol (1 – 50 μM) | LPS (1 μg/ml) | - NO assay with Griess reagent<br>- Electrophoresis<br>- Western blot<br>- RT-PCR<br>- Densitometric analysis | - Expression of iNOS mRNA ↓<br>- TLR4 expression ↓<br>- Phosphorylation and degradation IkB complex ↓ | Panaro et al. (2013) |
| Caco-2/ RAW264.7 | Luteolin (100 μM) | LPS (1 ng/mL) | - RT-PCR<br>- MTS assay<br>- Immunofluorescence staining of NF- κB p65 | - IL-8 mRNA expression in Caco-2 cells ↓<br>- Expression pro-inflammatory cytokines mRNA (TNF-α, IL -6, IL-1β) in RAW264.7 ↓ | Nishitani et al. (2013) |
| HEK293 + THP-1 | Apigenin (6,25-25 μM) | LPS (10 - 100 ng/ml) | - Immune response PrimerPCR assay<br>- Quantitative RT-PCR<br>- ELISA<br>- Western blot<br>- Immunofluorescence staining<br>- Measurement of NF-κB activity | - Production of IL-6, IL-1β, and TNF-α ↓<br>- Production of IL-10 ↑<br>- ERK1/2 activation ↓<br>- NF-κB activation ↓ | Zhang et al. (2014) |
| HT-29 human colonic epithelial | Quercetin 3,7-O-dimethyl ether (10–100 μM) | LPS (1 μg/ml) | - NO assay<br>- ELISA<br>- Western blot | - Expression of iNOS and COX-2 ↓<br>- Production of IL-8 ↓ | Lee et al. (2016) |
| Human aortic endothelial cells (HAEC) | Quercetin (5–20 μM) | LPS (100 ng/ml) | - ROS assay via DCFH2-DA<br>- ELISA<br>- Immunoblotting | - mRNA and protein expression of E-selectin and ICAM-1 ↓<br>- ROS levels ↓<br>- Quercetin did not inhibit activation NF-κB<br>- Quercetin induced activation of Nrf2 and p38 MAP kinase, upregulating mRNA and protein expression of the antioxidant enzymes, HO-1, NAD(P)H dehydrogenase and glutamate-cysteine ligase. | Li et al. (2016) |

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|---|---|---|---|---|---|
| HUVEC | Hyperoside (10-50µmol/L) | LPS (1 µg/ml) | - Quantitative RT-PCR<br>- ELISA<br>- Western Blot | - Expression of IL-1β, IL-6, TNF-α and iNOS mRNA ↓<br>- Expression of TLR-4 and phosphorylation of IκBα and p65 ↓ | Zhou et al. (2018) |
| HUVEC | Tricin (1,5-30 µM) | LPS (1 µg/ml) | - ELISA<br>- RT–PCR<br>- Flow cytometry | - Activation of TNF-α, IFN-γ and MCP1 ↓<br>- Activation of ICAM-1, VCAM-1 and E-Selectin ↓ | Shalini et al. (2016) |
| HUVEC | Cyanidin-3-O-Glucoside (12,5-50 µg/mL) | LPS (1 µg/mL) | - ELISA<br>- Western blot | - Induction TNF-α, IL-1β, and IL-6 ↓<br>- Activation NF-κB and MAPK signaling pathways ↓<br>- Phosphorylation of IκB-α, NF-κB/p65, ERK, p38 MAPK, and JNK ↓ | Ma et al. (2015) |
| HUVEC | Orientin + isoorientin (5-50µM) | LPS (100 ng/ml) | - ELISA<br>- Western blot | - Activation ERK 1/2 ↓ | Bae et al. (2015) |
| HUVEC | Wogonin (1-100µM) | LPS (1 µg/mL) | - Immunofluorescence staining<br>- Transendothelial cell migration assay<br>- Western blot | - Vascular permeability HUVEC cells ↓<br>- Expression of junctional proteins VE-Cadherin, Claudin-5 and ZO-1 ↑<br>- Expression of TLR4, p-PLC, p-MLCK and p-MLC ↓ | Huang et al. (2015) |
| HUVEC + THP-1 | Orientin, isoorientin, vitexin and isovitexin (1-40µM) | LPS (100 ng/ml) | - Permeability assay using Evans blue bound albumin<br>- ELISA<br>- RT-PCR<br>- Leukocyte adhesion assay<br>- ROS levels via DCFH2-DA | - Expression of CAMs ↓<br>- Adhesion and migration of leukocytes ↓<br>- LPS-induced EPCR shedding ↓<br>- Production of TNF-α and IL-6 ↓<br>- Activation of NF-κB and ERK ½ ↓<br>- Production iNOS ↓<br>- Expression of TLR-4 ↓ | Lee et al. (2014) |
| HUVEC + THP-1 | Quercetin (1-20µM) | LPS (1 µg/mL) | - Enhanced Chemoluminescence Plus kit<br>- Luciferase reporter assay<br>- Monocyte adhesion assay | - ICAM-1 and VCAM-1 expression ↓<br>- Quercetin did not affect the IκBα degradation stimulated with LPS<br>- NF–kB activity ↓<br>- Adhesion of THP–1 to HUVECs ↓ | Cho et al. (2016) |

| | | | | | |
|---|---|---|---|---|---|
| HUVEC + THP1 | Baicalin, baicalein and wogonin (1-100µM) | LPS (100 ng/ml) | - Monocyte adhesion assay<br>- Migration assay with Evans blue-bound albumin<br>- ELISA | - Expression of CAMs ↓<br>- Adhesion/transendothelial migration of monocytes to HUVEC ↓<br>- Production TNF-α and IL-6 ↓<br>- Activation NF-κB and ERK 1/2 ↓<br>- TLR-4 expression ↓ | Lee et al. (2015) |
| RAW 264.7 | Quercetin-3-O-glucuronide (20µM) | LPS (1 µg/mL) | - Zymography<br>- Immunoblotting<br>- HPLC-ECD analysis<br>- LC-MS/MS analysis | - The accumulation of the deconjugated quercetin derivatives was induced in the LPS-activated macrophages.<br>- JNK activation ↓<br>- Nuclear translocation and phosphorylation of NF-κB was not affected | Ishisaka et al. (2013) |
| RAW 264.7 | Fisetin (0-30µM) | LPS (10 - 100 ng/ml) | - Immunoblotting<br>- RT-PCR<br>- NO assay with Griess reagent | - Release NO ↓<br>- iNOS, TNF-α and COX-2 mRNA levels ↓<br>- Nuclear translocation of p65/nuclear factor NF-κB ↓ | Kim et al. (2015) |
| RAW 264.7 | Dihydromyricetin (37,5-300 µM) | LPS (1 µg/mL) | - NO assay with Griess reagent<br>- Western blot<br>- ELISA | - Production of nitric oxide (NO) ↓<br>- Levels of TNF-α, IL-1β, and IL-6 ↓<br>- Level of IL-10 ↑ | Hou et al. (2015) |
| RAW 264.7 | Dihydromyricetin (10-100 µM) | LPS (100 ng/ml) | - Western blot<br>- High resolution Atomic Force Microscope (AFM)<br>- Quantitative RT-PCR<br>- Nitrite assay<br>- ELISA | - Morphological change and membrane alterations of macrophages ↓<br>- NO secretion ↓<br>- iNOS and COX-2 protein expression ↓<br>- p65 phosphorylation ↓ via suppression of IKKβ activity and IKKα/β phosphorylation | Wang et al. (2016) |
| RAW 264.7 | Luteolin + luteolin-7-O-glucoside (5-50µM) | LPS (1 µg/ml) | - NO assay<br>- Enzyme immunoassay (EIA) kit<br>- Western blot | - NO and PGE2 ↓<br>- iNOS and COX-2 ↓<br>- Activation NF-κB and AP-1 ↓<br>- Luteolin decreases LPS-induced inflammation better than luteolin-7-O-glucoside | Park et al. (2013) |

| | | | | | |
|---|---|---|---|---|---|
| RAW 264.7 | Kaempferol (50-100µM) | LPS (1 µg/mL) | - NO assay<br>- Quantitative RT-PCR | - NO and PGE2 ↓ | Kim et al. (2015) |
| RAW 264.7 | Prunetin (25-100 µM) | LPS (1µg/ml) | - NO assay<br>- ELISA<br>- Western blot<br>- RT-PCR<br>- NF-κB transcription factor assay<br>- Plasmid, transient transfection, and luciferase assay | - iNOS and COX-2 expression ↓<br>- TNF-α, IL-6 and IL-1β expression ↓<br>- NF-κB activation ↓ | Yang et al. (2013) |
| THP-1 | Quercetin, naringenin, epicatechin, hesperetin, peonidin-3-glucoside (P3G) and C3G (0.1-10 µM) | LPS (100 pg/mL- 1µg/ml) | - ELISA<br>- Nanodrop ND-1000 spectrophotometer.<br>- RT-PCR<br>- NF-κB luciferase reporter assay | - TNF-α secretion ↓ (not all flavonoids significant reduction)<br>- None significantly inhibited IL-1β and IL-10 secretion<br>- None had any effect on NF-κB reporter activity | di Gesso et al. (2015) |

## Appendix 3: Studies on the influence of flavonoids on cellular stress induced by mitochondrial stressors in various cell lines

*Table 6- Studies on the influence of flavonoids on cellular stress induced by mitochondrial stressors in various cell lines*

| Cell line | Flavonoid (concentration) | Stress factor (concentration) | Methods used in study | Result of addition flavonoid to stressed cell line compared to stressed cell without the flavonoid | Reference |
|---|---|---|---|---|---|
| Caco-2 | Quercetin (25-100 µM) | Valinomycin (0,02- 0,1 µM) | - MTT assay<br>- ROS assay<br>- Flow cytometry<br>- Cellular flavonoid localization by using DiI stain<br>- Confocal microscopy | - Intracellular ROS content ↓<br>- Cell viability of Caco-2 cells ↑ compared to valinomycin-only treatment.<br>- Intracellular accumulation of O-methylated quercetin metabolites compared to cells treated only with quercetin ↑<br>- Valinomycin-untreated cells: quercetin and O-methylated quercetin metabolite localized in cell membrane ↔ valinomycin treatment: uptake by the cells | Gonzales et al. (2016) |
| Adipocytes | Quercetin (5-100 µM) and apigenin (5-100 µM) | Tunicamycin (2 µg/ml) | - MTT Assay<br>- ROS assay<br>- MitoTracker Green FM | - Depolarization transmembrane potential change ↓<br>- Mitochondrial mass reduction ↓ | Nisha et al. (2014) |
| EA.hy926 | Delphinidin (10mg/l) | Oligomycin (1 µM) + FCCP (0,2–1 µM) | - Basal respiration rate of cells was determined by measuring the linear rate of oxygen consumption,<br>- Addition oligomycin to determine the non-phosphorylating respiration rate<br>- Stepwise addition of FCCP to record the uncoupling respiration rate | - Delphinidin did not affect basal or FCCP respirations but significantly decreased oligomycin respiration. | Duluc et al. (2014) |
| MDCK kidney cells (not human) | Diosmetin (10µM) | Oligomycin (15 µM) | - Bioluminescent ATP determination<br>- HPLC-UV<br>- ROS assay | - Addition of oligomycin reduces the ATP concentration compared to the untreated cells<br>- Addition of the flavonoid has no effect on the ATP reduced concentration<br>- Addition of the flavonoid to cells not treated with oligomycin increases the ATP concentration compared to the untreated cells | Poór et al. (2014) |

| Human chondrocytes | Reservatrol (50-250 µM) | Oligomycin (20 µg/ml) + TNF-α (5 ng/ml) | - Flow cytometry analysis<br>- ELISA<br>- Chemotaxis assays | - PGE2, COX-2 and IL-8 levels ↑ | Vaamonde-Garcia et al. (2012) |
|---|---|---|---|---|---|
| Caco-2 | Quercetin (0.01 mg/ml), resveratrol (0.1 mg/ml) and rutin (1 mg/ml) | Indo-methacin (250 µM) | - Intracellular ATP levels were quantified using the CellTiter-Glo® kit<br>- Mitochondrial membrane potential (MMP) using CMX-Ros<br>- Complex I, II and II activity from isolated mitochondria<br>- Superoxide radical production<br>- HPLC | - Decrease in ATP levels induced by INDO ↓<br>- Quercetin was the most efficient of all the tested flavonoids<br>- Decrease in MMP induced by INDO ↓<br>- Quercetin protects against complex I alterations induced by INDO<br>- Direct interaction between quercetin and indomethacin is ruled out | Carrasco-Pozo et al. (2012) |
| Isolated mitochondria of rat brain and heart | Quercetin (1-10µM), kaempferol (1-10µM) | Rotenone (10 µM), antimycin A (2 µM) | - Production of $H_2O_2$ by mitochondria in respiration buffer by the Amplex Red method | - Production $H_2O_2$ by mitochondria ↓, even when $H_2O_2$ production rate was stimulated by the mitochondrial inhibitors | Lagoa et al. (2011) |

# APPENDIX 4: SRB data cytotoxicity assay

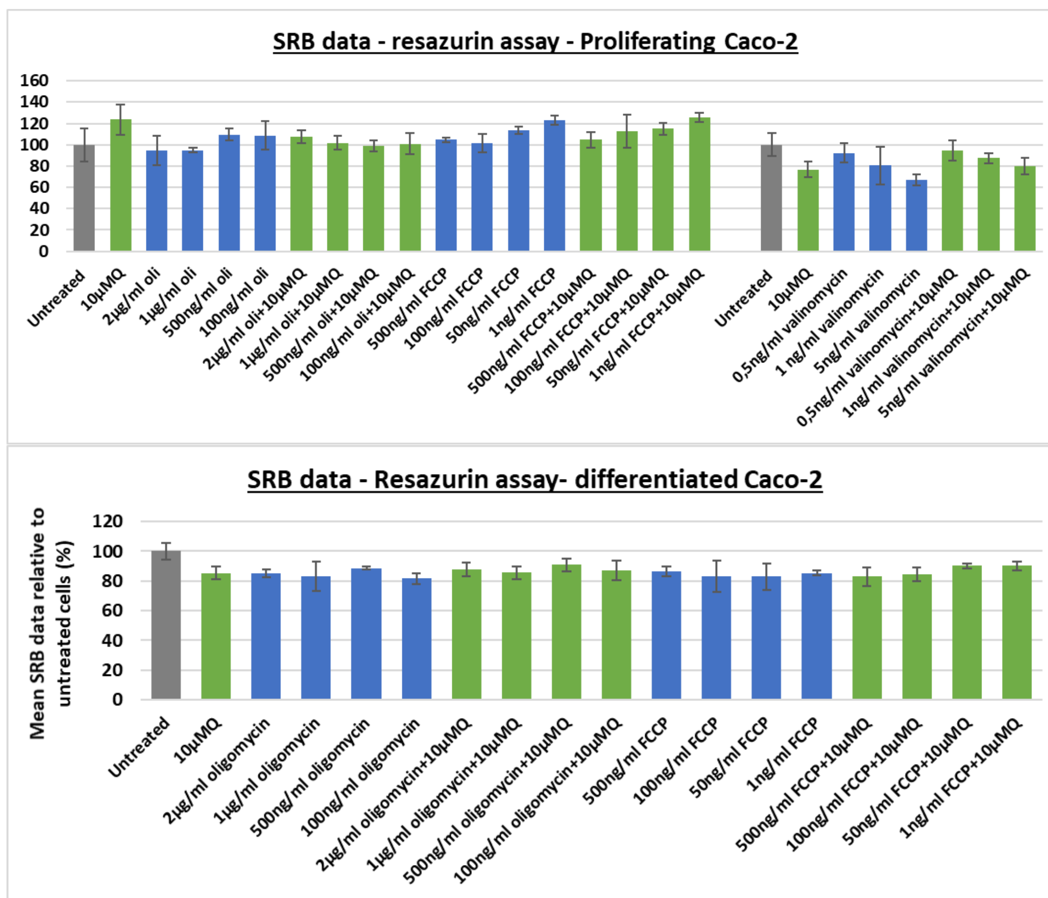


*Figure 28: SRB data of cytotoxicity assay of mitochondrial stressors on proliferating and differentiated Caco-2 cells. Error bars indicate the standard deviation of biological replicates (N= 6).*

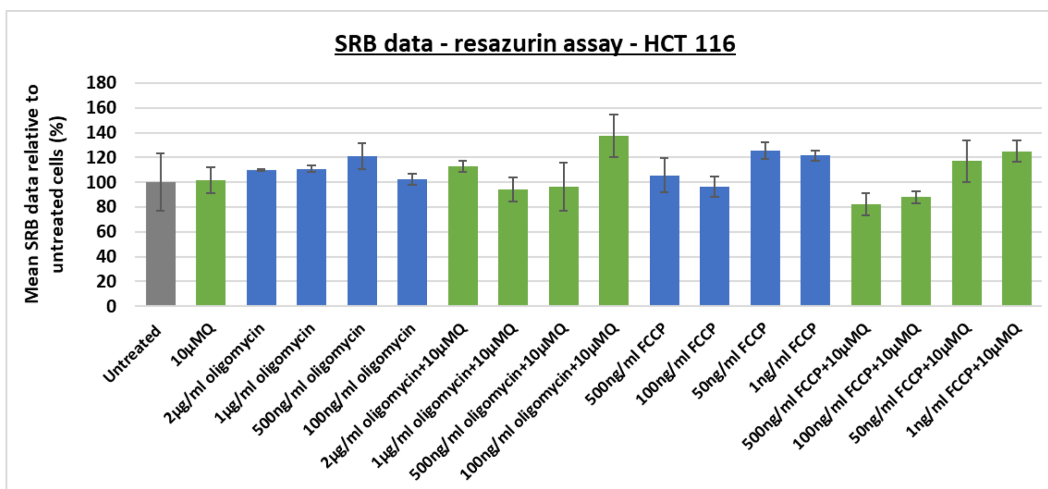


*Figure 29:SRB data of cytotoxicity assay of mitochondrial stressors on HCT 116 cells. Error bars indicate the standard deviation of biological replicates (N= 6).*

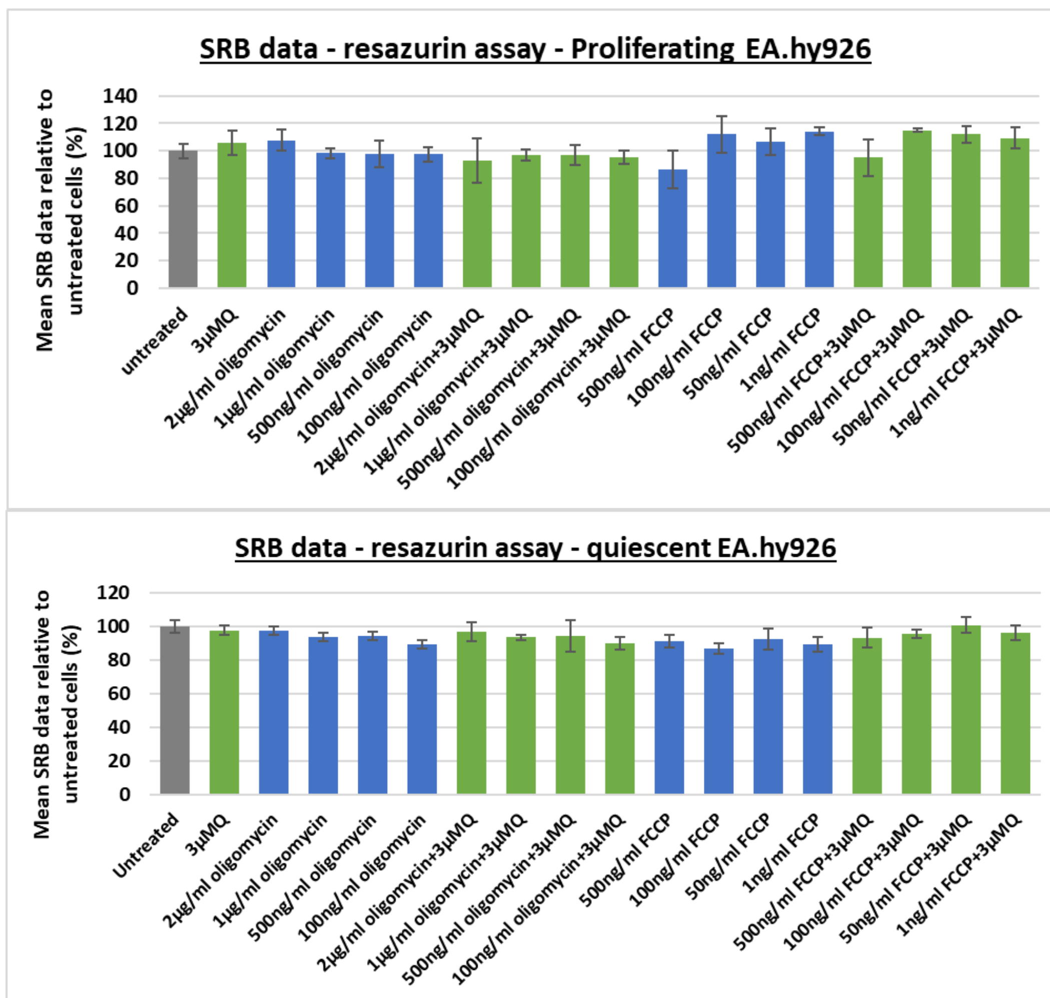


*Figure 30: SRB data of cytotoxicity assay of mitochondrial stressors on proliferating and quiescent EA.hy926 cells. Error bars indicate the standard deviation of biological replicates (N= 6).*

# APPENDIX 5: SRB data accumulation assay

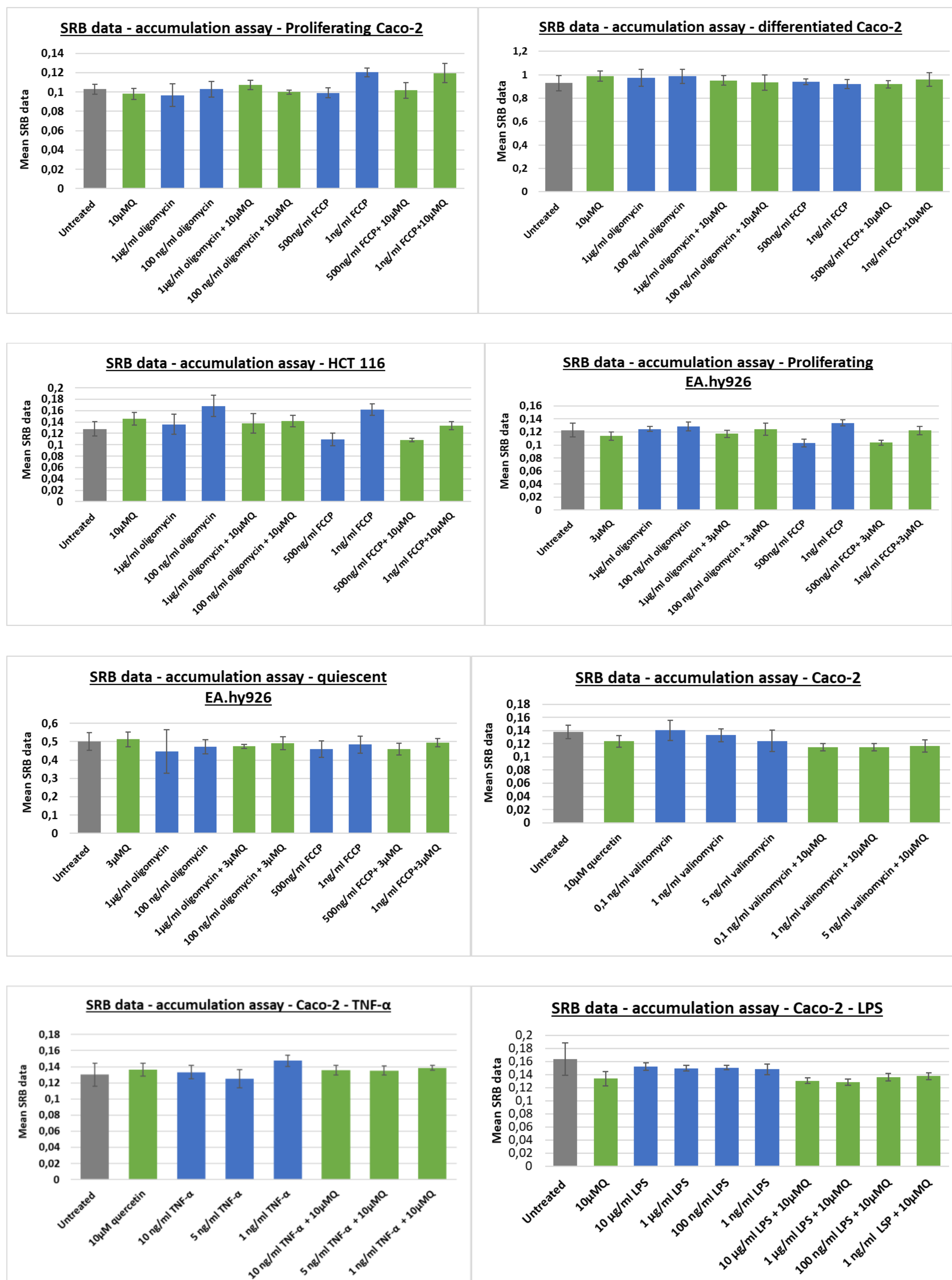


*Figure 31: SRB data of accumulation assay for different cell lines and stressors. Error bars indicate the standard deviation of biological replicates (N= 6).*