FACULTY OF BIOSCIENCE ENGINEERING

The effect of quercetin on *in vitro* cell models for endothelial dysfunction

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PREAMBLE: THE IMPACT OF COVID-19 MEASURES ON THIS MASTER THESIS

On March 12, 2020, the government decided to take measures in order to prevent spreading of Covid-19 in Belgium. Subsequently, Ghent University urged students and personnel to respect social distancing at all times. Since the measures became stricter each day, it was decided that from the 19th of March 2020, access to the lab was prohibited for master students, which meant that all lab work was immediately discontinued. Due to my Erasmus exchange to Uppsala University in Sweden during the first semester of this academic year, I only started working in the lab from the 6th of January 2020. Therefore, the time I could work in the lab was unexpectedly reduced, implicating that the results in this master thesis were obtained during a period of approximately 2.5 months. Due to the unexpected circumstances concerning Covid-19, some experiments could not be executed as planned. More specifically, due to time restriction because of Covid-19, it was not possible to determine, using ELISA assays, pro-inflammatory markers in the different compartments of the triple co-cultures and the development of triple co-culture model C (Figure 9) was cancelled. As a replacement, hypotheses regarding these experiments are given, based on findings in literature. As a result, the theoretical aspect occupies a large part of this master thesis. Moreover, some experiments could only be performed once, so one has to be careful about making strong conclusions about the observed results.

This preamble was drawn up after consultation between the student and the supervisor and is approved by both.

ABSTRACT

Atherosclerosis is caused by vascular endothelial dysfunction and can lead to a range of cardiovascular diseases, thereby contributing to one of the greatest causes of death worldwide. Quercetin, i.e. a polyphenol that belongs to the flavonoid subfamily, is found in a plant-based diet and is reported to provoke beneficial effects on the prevention of atherosclerosis due to its anti-oxidant and anti-inflammatory properties.

Although the bioactivity of quercetin has been explored extensively in literature, many *in vitro* studies do not take the low bioavailability and extensive metabolism of quercetin into account. In this study, the impact of psychological stressors (adrenaline and cortisol) on quercetin bioavailability was first investigated using monocultures of intestinal and endothelial cells. Second, novel triple co-culture models for endothelial dysfunction were developed including intestinal cells, endothelial cells and monocytes/macrophages in order to study the influence of quercetin towards endothelial dysfunction under physiological relevant conditions.

The findings of the monoculture studies show that the intracellular quercetin accumulation in intestinal cells increased upon adrenaline stress, but decreased upon cortisol stress. This suggests that cellular stress has the ability to modulate the *in situ* intracellular quercetin concentration and potential bio-efficacy.
Monocyte recruitment and adhesion to a (stressed) endothelium is essential for the onset of atherosclerosis. Using a triple co-culture model, it was observed that LPS-induced stress enhanced monocyte adhesion to endothelial cells by inducing endothelial dysfunction. Interestingly, this was counteracted by co-administration with quercetin. This finding illustrates the potential of quercetin (metabolites) to protect against endothelial dysfunction and prevent the early stages of atherosclerosis.

In the search for prevention of endothelial dysfunction and atherosclerosis, the use of novel *in vitro* triple co-culture models can facilitate to give new perspectives to unravel the mechanistic pathways of quercetin (metabolites).

SAMENVATTING

Atherosclerose wordt veroorzaakt door vasculaire endotheel disfunctie en kan leiden tot diverse hart- en vaatziekten, waardoor het bijdraagt tot één van de grootste doodsoorzaken wereldwijd. Quercetine, i.e. een polyfenol dat tot de flavonoïde subfamilie behoort, is aanwezig in een plantaardig dieet en zou gunstige effecten hebben op het voorkomen van atherosclerose door haar anti-oxidant en anti-inflammatoire eigenschappen.

Hoewel de bio-activiteit van quercetine reeds grondig onderzocht is in de literatuur, houden veel *in vitro* studies geen rekening met de lage bio-beschikbaarheid en het uitgebreid metabolisme van quercetine. In deze studie werd allereerst het effect van psychologische stressoren (adrenaline en cortisol) op de bio-beschikbaarheid van quercetine onderzocht met behulp van monoculturen van intestinale cellen en endotheelcellen. Daarnaast werden er nieuwe triple co-cultuur modellen voor endotheel disfunctie ontwikkeld, bestaande uit intestinale cellen, endotheelcellen en monocyten/macrofagen, om de invloed van quercetine op endotheel disfunctie te bestuderen onder fysiologisch relevante condities.

De bevindingen van de monocultuur studies tonen aan dat de intracellulaire quercetine accumulatie in intestinale cellen toenam bij adrenaline stress, maar afnam bij cortisol stress. Dit suggereert dat cellulaire stress het vermogen heeft om de *in situ* intracellulaire quercetine concentratie en potentiële bio-werkzaamheid te moduleren.
Monocyt rekrutering en adhesie aan een (gestresseerd) endotheel is essentieel voor het ontstaan van atherosclerose. Door middel van een triple co-cultuur model werd waargenomen dat LPS-geïnduceerde stress monocyt adhesie aan endotheelcellen verhoogt door endotheel disfunctie te veroorzaken. Dit werd tegengewerkt door gelijktijdige toediening met quercetine. Deze bevinding illustreert het potentieel van quercetine (metabolieten) om te beschermen tegen endotheel disfunctie en de vroege stadia van atherosclerose te voorkomen.

In de zoektocht naar de preventie van endotheel dysfunctie en atherosclerose, kan het gebruik van nieuwe *in vitro* triple co-cultuur modellen bijdragen tot nieuwe perspectieven om de mechanistische pathways van quercetine (metabolieten) te ontrafelen.

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LIST OF ABBREVIATIONS

AP-1	Activator protein 1
apoA-1	Apolipoprotein A1
ATP	Adenosine triphosphate
BCRP	Breast cancer resistant protein
BH_4	Tetrahydrobiopterin
cAMP	Cyclic adenosine monophosphate
COMT	Catechol-O-methyltransferase
DMEM	Dulbecco's Modified Eagle's Medium
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
FBS	Fetal bovine serum
GLUT	Glucose transporter
HDL	High density lipoprotein
HO-1	Heme oxygenase 1
IFN-γ	Interferon γ
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JAM	Junctional adhesion molecules
Keap	Kelch-like ECH-associated protein
LPH	Lactase-phlorizin hydrolase
LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant protein
MRP	Multidrug resistant protein
MyD88	Myeloid differentiation factor 88
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
NEAA	Non-essential amino acids
NF-κB	Nuclear transcription factor κB
NO	Nitric oxide
NQO1	NAD(P)H quinone 1 dehydrogenase
Nrf2	Erythroid 2-related factor 2
OATP	Organic anion transporting polypeptide
PBS	Phosphate buffered saline
PECAM	Platelet endothelial cell adhesion molecules
P-gp	P-glycoprotein
PMA	Phorbol-12-myristate-13-acetate
ROS	Reactive oxygen species
SGLT	Sodium-dependant glucose transporter
SRB	Sulforhodamine B
SULT	Sulfotransferase
TEER	Transepithelial/transendothelial electrical resistance
TLR4	Toll-like receptor 4
TNFR	TNF receptor
TNF-α	Tumour necrosis factor α
UGT	UDP-glucuronyl transferase
vWF	von Willebrand Factor
(ox)LDL	(Oxidized) low density lipoprotein
(s)ICAM	(Soluble) intracellular adhesion molecule
(s)VCAM	(Soluble) vascular cell adhesion molecule

INTRODUCTION

Atherosclerosis can lead to a range of cardiovascular diseases and contributes thereby to one of the greatest causes of death worldwide. It is associated with endothelial dysfunction which is characterized by a pro-oxidative and pro-inflammatory state of the endothelium. The pathogenesis of atherosclerosis will be discussed in the literature study (section 1.1).

Quercetin is a polyphenol belonging to the flavonoid subfamily and is found in several plant-based food products. It is reported to possess anti-inflammatory and anti-oxidant properties which may be beneficial in the prevention of endothelial dysfunction and atherosclerosis. Epidemiological studies and clinical trials demonstrate that health status is a crucial factor in the responsiveness of subjects to quercetin (section 1.3). Moreover, there could be a correlation between health status and the large inter-individual variability in quercetin bioavailability.

In order to unravel the mode of action of quercetin in both diseased and healthy conditions *in vivo*, *in vitro* studies, using stressors to simulate a diseased state, can serve as a tool to better understand the mechanistic pathways. Several *in vitro* studies using quercetin are described in literature (section 1.4), however, most of these studies did not take quercetin bioavailability and biotransformation into account. After all, quercetin is extensively metabolized in the intestine and liver before reaching peripheral cells. The absorption and metabolism of quercetin will be discussed in section 1.2.

In this study, it is investigated whether cellular stress, induced by the stressors adrenaline and cortisol, can modulate the absorption of quercetin. In addition, novel cell models for endothelial dysfunction, including intestinal, endothelial and immune cells, are developed. Using these *in vitro* models, bioactivity of quercetin towards endothelial dysfunction, induced by the stressor LPS, can be studied under physiological relevant conditions.

1. LITERATURE STUDY

1.1 An introduction to the pathogenesis of atherosclerosis

Atherosclerosis is a chronic inflammatory disease that is associated with vascular endothelial dysfunction and is characterised by plaque formation causing gradual narrowing of the vascular lumen. It can lead to a range of vascular disorders e.g. coronary artery disease, carotid artery disease and peripheral arterial disease and is thereby responsible for one of the greatest causes of death worldwide (Shoenfeld, Sherer, & Harats, 2001). Approximately 1 in 3 people die from cardiovascular diseases every year (WHO, 2019). Especially the combination with platelet-mediated thrombotic events – due to erosion or rupture of atherosclerotic plaques – can be fatal (Bennett, Sinha, & Owens, 2016). A plaque consists of a necrotic lipid core – derived from death foam cells, endothelial cells, smooth muscle cells, inflammatory and immune cells –, connective tissue and a fibrous cap encapsulating the plaque. Fatty streaks, i.e. lipid accumulation on the vascular walls that can evolve into atheromatous plaques over time, are already observed in early childhood and the first clinically relevant lesions appear in middle-aged adults (Marchio et al., 2019). Risk factors include high levels of cholesterol, hypertension, diabetes and smoking and it is demonstrated that men are more susceptible than women. High levels of high density lipoprotein (HDL), apolipoprotein A1 (apoA-I), exercise and alcohol[1] are protective factors.

Since vascular endothelial dysfunction is associated with the onset of the pathogenesis of atherosclerosis, the structure of the vascular wall will be discussed in section 1.1.1. In addition, three type of cells are principally involved in the atherogenic process: endothelial cells, macrophages and smooth muscle cells (Falk, 2006). The role of each of these cells will be uncovered in sections 1.1.2-1.1.5.

1.1.1 The structure of the vascular wall

The vascular wall is composed of different layers that will be discussed in this section according to the structural order as illustrated in Figure 1. The first layer, the tunica intima, is in contact with the lumen and consists of a monolayer of endothelial cells. This monolayer functions as a semi-permeable barrier that regulates the passage of molecules between the blood and tissues (Mazurek et al., 2017). Transport can occur transcellular, through the membrane of the endothelial cells, or paracellular, via the cell junctions.

The endothelial cells rest on a basement membrane or basal lamina, which is part of the extracellular matrix and contains i.a. laminin, type IV collagen, fibronectin, nidogen and proteoglycans. It offers structural support, regulates endothelial cell migration and proliferation and provides an environment for cellular interaction (LeBleu, Macdonald, & Kalluri, 2007; Marchio et al., 2019; Mazurek et al., 2017).

The internal elastic lamella is a fenestrated layer of elastic tissue that separates the tunica intima from the tunica media. The fenestrae mediate the transport of macromolecules from the intima to the media

[1] The atheroprotective effect of alcohol is dose-dependent: intake of low alcohol concentrations decelerates atherogenesis, whereas high concentrations are detrimental (Kiechl et al., 1998).

(Tada & Tarbell, 2004). The tunica media comprises mainly smooth muscle cells alternated with elastic lamellae and collagen bundles in between (Mazurek et al., 2017). Vascular smooth muscle cells can be subdivided into synthetic or contractile phenotypes according to their function. Contractile smooth muscle cells are predominant in normal conditions where they regulate the blood vessel diameter by contraction and relaxation. In an inflammatory context, the contractile cells can be converted to a less differentiated state, namely synthetic cells which are able to proliferate and migrate and are responsible for the production of extracellular matrix proteins among which collagen and elastin (Metz, Patterson, & Wilson, 2012).

Analogous to the internal elastic lamella, the external elastic lamella or lamina forms the separation between the tunica media and the outermost layer, the tunica externa, and provides structural support (Pugsley & Tabrizchi, 2000). The tunica externa is made of fibroelastic connective tissue, nerve endings, adipose tissue, lymphatics, pericytes, fibroblasts and immune cells, more specifically dendritic cells, lymphocytes, macrophages and mast cells (Grant & Twigg, 2013; Marchio et al., 2019). Attributable to its complexity and heterogeneity, the tunica externa is involved in the regulation of the vascular structure and function (Stenmark et al., 2013). In large blood vessels, this layer is also stored with vasa vasorum, i.e. small blood vessels that take care of the supplementation of nutrients and oxygen, which penetrate the outer layers of the tunica media as well (Boyle, Sedding, & Haverich, 2017).

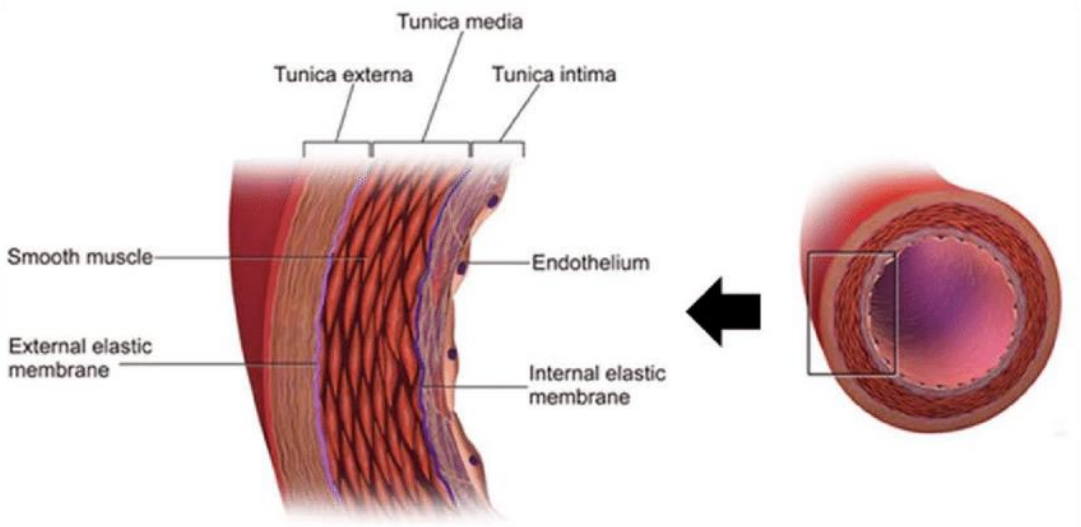


Figure 1. The structure of the vascular wall (Weidmann et al., 2015)

1.1.2 The involvement of the vascular wall in the pathogenesis of atherosclerosis

1.1.2.1 Endothelial cells

Endothelial cells have atheroprotective properties due to the production of nitric oxide (NO), which is a vasodilator (Marchio et al., 2019). As Figure 2 schematically summarizes, NO is derived from L-arginine by endothelial nitric oxide synthase (eNOS), an enzyme that is located in endothelial caveolae, i.e. invaginations of the plasma membrane (Davignon & Ganz, 2004). For activation of eNOS, a broad range of cofactors – nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme and calcium-dependant calmodulin – is required (Shimokawa, 1999). As depicted in Figure 2, the eNOS-derived NO diffuses to the vascular smooth muscle cells where it activates guanylate cyclase (GC), i.e. an enzyme that synthesizes cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP), leading to relaxation of smooth muscle cells and eventually vasodilation. Other functions of NO include the inhibition of platelet aggregation, leukocyte infiltration and smooth muscle cell proliferation and the prevention of oxidative modification of low density lipoproteins (LDL), i.e. particles transporting cholesterol from the liver to the tissues (Davignon & Ganz, 2004; Shimokawa, 1999).

Vascular endothelial dysfunction is a condition that is associated with impaired endothelial function and is known to be involved in the initiation and progression of atherosclerosis. Vascular endothelial dysfunction is characterized by reduced vasodilation – due to downregulation of NO and upregulation of the endothelin-1 vasoconstrictor –, increased endothelial permeability and the generation of a prothrombic and pro-inflammatory state (Endemann & Schiffrin, 2004). As illustrated in Figure 3, risk factors of atherosclerosis, i.e. smoking, diabetes, hypertension and hypercholesterolemia, are often associated with vascular endothelial dysfunction and an increased production of reactive oxygen species (ROS) by endothelial cells, vascular smooth muscle cells and external cells, especially at sites with reciprocating shear stress (Harrison, Griendling, Landmesser, Hornig, & Drexler, 2003). ROS are a class of chemical reactive substances that cover a variety of molecules containing oxygen such as superoxide anion ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and hypochlorous acid (HOCl) (Cai & Harrison, 2000). These reactive oxygen species have a broad field of action: upregulation of the adhesion molecule expression, stimulation of smooth muscle cell proliferation and migration, activation of matrix metalloproteinases, apoptosis in the endothelium and creation of oxidative stress (Harrison et al., 2003). Enzymatic sources of ROS comprise mitochondrial respiration, xanthine oxidase, NAD(P)H oxidase and NOS which are responsible for the increased production of ROS in response to specific stimuli, e.g. interferon γ (IFN-γ) which upregulates the expression and activity of xanthine oxidase (Cai & Harrison, 2000).

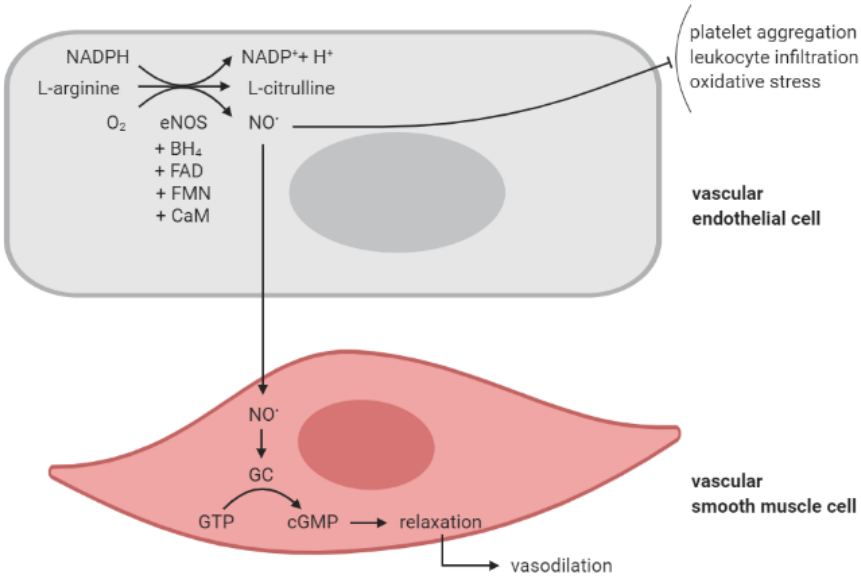


Figure 2. NO production and action mechanism in the vascular wall

Endothelial dysfunction is characterized by an impaired production or activity of NO, caused by a decreased expression or activation of eNOS, unavailability of eNOS cofactors and/or an enhanced NO degradation by ROS. After all, superoxide anion is able to react with NO which leads to the formation of peroxynitrite ($ONOO^-$) and thus NO inactivation (Cai & Harrison, 2000). This reaction can be overcome by superoxide dismutase (SOD), i.e. an enzyme that scavenges superoxide by converting $O_2^{-\cdot}$ and H^+ to H_2O_2 and O_2 (Chavez, Lakshmanan, & Kavdia, 2007). In physiological conditions, the interaction between NO and $O_2^{-\cdot}$ is also minimized by endogenous anti-oxidants (Cai & Harrison, 2000). As mentioned before, eNOS is not only capable of producing NO, it is also a potential source of ROS. In case of a depletion of BH_4 – e.g. when it is oxidized in the presence of a low level of ROS – or L-arginine, eNOS uses molecular oxygen as a substrate for the production of $O_2^{-\cdot}$ and H_2O_2. This process is described as the uncoupling of eNOS (Cai & Harrison, 2000; Harrison et al., 2003). Increased levels of ROS combined with NO bioavailability impairment promote oxidative stress which encourages the oxidation of LDL, resulting in an accumulation of oxidized LDL (oxLDL) in the intima of the vascular wall (Balakumar, Koladiya, Subbiah, Rathinavel, & Singha, 2008). OxLDL has a key role in the processes associated with plaque forming. It acts as a chemokine and thereby promotes the recruitment of monocytes. Furthermore, oxLDL can cause the transition of fatty streaks to more complex plaques by inducing the cell death of macrophages, due to its cytotoxic properties (Baird, Hampton, & Gieseg, 2004; Napoli et al., 1997). The contribution of oxLDL in the formation of atheromatous plaques will be discussed more in detail in section 1.1.4.

Risk factors of atherosclerosis do not only induce a shift of endothelial cells towards a pro-oxidative state, they also promote a pro-inflammatory state, as depicted in Figure 3. Stimuli like chronic inflammation (tumour necrosis factor α, TNF-α), bacterial infections (lipopolysaccharide, LPS), hypercholesterolemia (LDL), hypertension (ROS) and disturbed shear stress target endothelial cells in lesion-prone areas and subsequently activate the endothelium by inducing a pathway including the

activation of nuclear transcription factor κB (NF-κB) (Gimbrone & García-Cardeña, 2016; Steyers & Miller, 2014). This is followed by an increased expression of adhesion molecules like intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin and P-selectin, cytokines like monocyte chemoattractant protein 1 (MCP-1) and prothrombic mediators like von Willebrand Factor (vWF) (Balakumar et al., 2008; Gimbrone & García-Cardeña, 2016). As described before, the vascular wall comprises endothelial cells, smooth muscle cells, macrophages and lymphocytes. Coordinated action of those cells generates a paracrine environment of pro-inflammatory cytokines, ROS and growth factors which sustain the chronic pro-inflammatory state (Gimbrone & García-Cardeña, 2016). In addition, vascular endothelial dysfunction can increase the permeability of the endothelial cells causing a higher transmission of LDL (Marchio et al., 2019). Specific pro-inflammatory stimuli, e.g. LPS and TNF-α, are able to modulate the permeability of the endothelial barrier. LPS and TNF-α can bind their corresponding endothelial receptors and thereby initiate a pathway which leads to the disassembly of inter-endothelial junctions, by the increase of cytosolic calcium concentration and activation of myosin light chain kinase (MLCK). The disruption of the junctions is associated with a higher endothelial permeability (Vandenbroucke, Mehta, Minshall, & Malik, 2008). Furthermore, upon binding of LPS and TNF-α, NF-κB is activated which induces the expression of caveolin-1, i.e. the structural protein of caveolae. This leads to an increased number of caveolae and an associated increase in transcellular endothelial permeability (Tiruppathi et al., 2008; Wang, Lim, Toborek, & Hennig, 2008).

Atherosclerosis is known as an inflammatory, oxidative disease, but also a focal disease, implying that the plaques appear at specific sites and not over the whole blood vessel. An explanation can be found in mechanotransduction, a process that is implicated in the initiation of the disease. It can be described as the conversion of mechanical forces that act on endothelial cells to alterations in biochemical pathways in the endothelial cells, causing conformational changes in i.a. adhesion complexes. Endothelial cells are subjected to different kinds of shear stress, according to the kind of blood flow. Laminar blood flow is associated with a sustained high shear stress and promotes downregulation of atherogenic genes, such as MCP-1, and upregulation of anti-oxidant genes, such as heme oxygenase 1 (HO-1) (X. L. Chen et al., 2003). It maintains the quiescent endothelial cell phenotype. In contrast, disturbed and reciprocating blood flow concur with low shear stress which is associated with a switch towards a pro-inflammatory endothelial cell phenotype (Figure 3) (Chistiakov, Orekhov, & Bobryshev, 2017). It contributes to atherosclerosis since it induces the expression of adhesion molecules, such as ICAM-1 and E-selectin, and the secretion of MCP-1 which promotes monocyte infiltration. Moreover, low shear stress induces endothelial apoptosis by regulating the balance between proapoptotic proteins, e.g. Bax, and anti-apoptotic proteins, e.g. Bcl-2 (Dong et al., 2017). To maintain vessel homeostasis, a high endothelial cell turnover rate is required (Xu, 2009). Besides the stimulation of the cell turnover, disturbed blood flow is associated as well with an increase in vascular endothelial growth factor (VEGF) expression which leads to increased vascular permeability and lipid uptake in endothelial cells. Oxidative stress and decreased NO generation are also linked to low shear stress (Marchio et al., 2019). Furthermore, low shear stress establishes a reduction in fenestrae of the internal elastic lamina by increased fibronectin expression and cell-matrix interactions. This event is associated with a decreased

internal elastic lamina permeability, resulting in a higher accumulation of macromolecules such as LDL in the vascular wall (Z.-Y. Guo, Yan, Bai, Zhang, & Jiang, 2008). Branch points are associated with disturbed and reciprocating blood flow, explaining the higher occurrence of atherosclerotic lesions near these sites (Marchio et al., 2019).

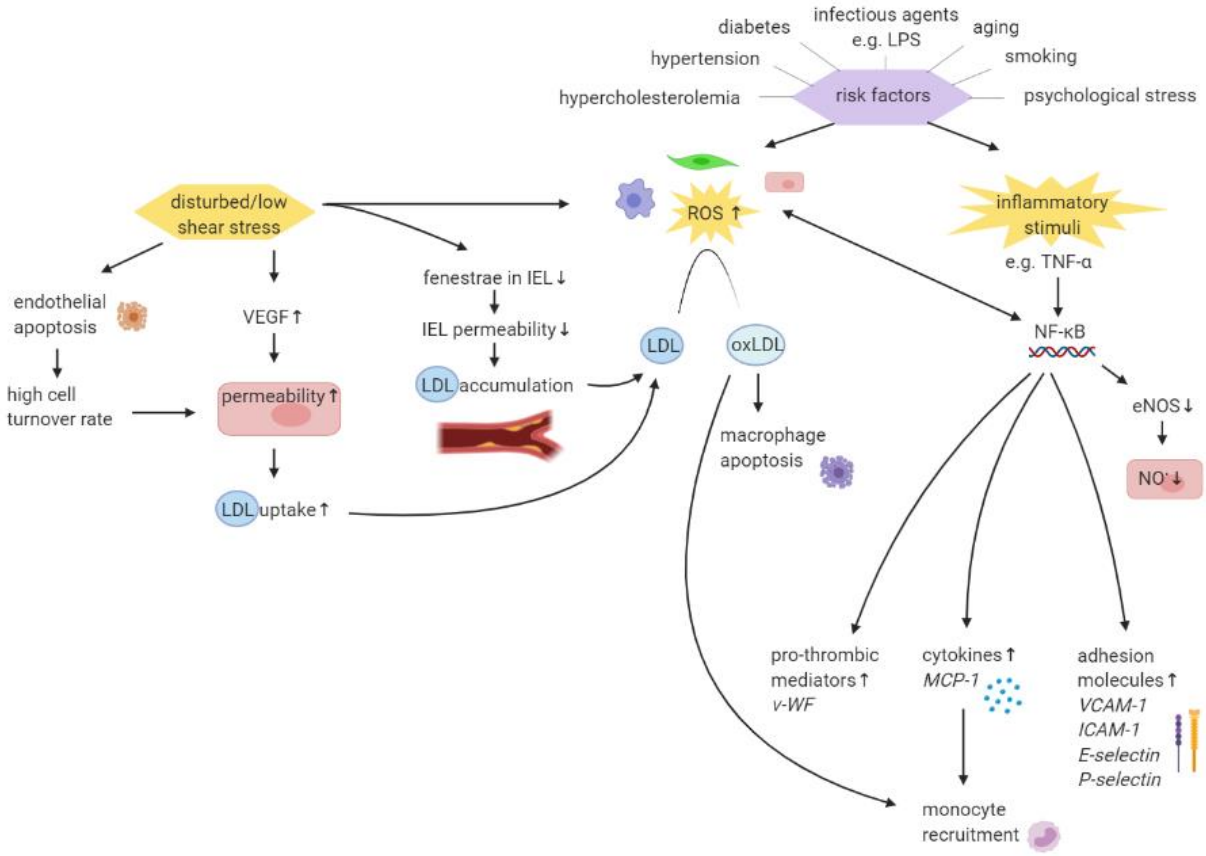


Figure 3. An overview of risk factors and their involvement in vascular endothelial dysfunction and atherosclerosis

1.1.2.2 Vascular smooth muscle cells

Smooth muscle cells do not come into play yet in the onset of atherosclerosis. They have a significant role later in the developing process of the disease because of their contribution to more advanced atherosclerotic lesions. Smooth muscle cells are located in the media layer of the vascular wall. They experience fluid shear stress, on the one hand by transmural interstitial flow, i.e. flow across the endothelial monolayer, which is driven by a transvascular pressure gradient, and on the other hand by direct exposure to the blood flow after vascular injury (Tarbell, Civelek, & Garanich, 2003). Laminar shear stress supresses smooth muscle cell proliferation and induces smooth muscle cell apoptosis (Fitzgerald et al., 2008; Ueba, Kawakami, & Yaginuma, 1997). In normal conditions, quiescent contractile smooth muscle cells are the most abundant phenotype. They regulate the width of the blood vessels by contraction or relaxation (Marchio et al., 2019). Studies have demonstrated that upon intimal

injury, contractile smooth muscle cells can dedifferentiate into the synthetic phenotype after which they migrate to the tunica intima and proliferate (Rudijanto, 2007). Phenotype switching occurs in response to diverse inputs from platelets (e.g. transforming growth factor β (TGF-β), platelet-derived growth factor-BB), endothelial cells, immune cells, plasma lipoproteins, hormones (e.g. angiotensin II) and inflammatory mediators (e.g. LPS) (Petsophonsakul et al., 2019; Strela et al., 2019; Toma & McCaffrey, 2012). It enables the performance of a broad range of tasks associated with normal cell physiology and disease, e.g. wound healing (D. Hu, Yin, Luo, Habenicht, & Mohanta, 2019). Synthetic smooth muscle cells are capable of producing extracellular matrix components like collagen, providing the initial plaque with a fibrous cap which is beneficial for the plaque stability. Phenotype switching can also generate macrophage-like cells, i.e. smooth muscle cells that have acquired macrophage properties and markers (Bennett, et al., 2016).

1.1.2.3 Vasa vasorum

Studies suggest that vasa vasorum dysfunction contributes to the initiation of atherosclerosis. Once initial plaques are formed, the rising demand for oxygen is met by neovascularization, i.e. the formation of new blood vessels. Due to the leakiness of immature neovessels, lipids and inflammatory mediators are deposed at the site of the plaque formation, thereby contributing to the progression of atherosclerosis (Boyle et al., 2017; de Vries et al., 2019; Parma, Baganha, Quax, & de Vries, 2017).

1.1.3 A confined overview of the different cells of the immune system concerning atherosclerosis

The immune system is a very complex organisation involving a wide variety of cells. The most prominent types of immune cells that are involved in the pathogenesis of atherosclerosis are introduced in this section and their specific role in the pathogenesis of atherosclerosis is discussed in section 1.1.4. However, it must be emphasized that several other types of immune cells, such as neutrophils, can be involved in this process as well. Here, the focus is mainly on monocytes and macrophages, since these cell types are used in the experimental part of this thesis.

1.1.3.1 Cells of the innate immune system

The first type of immune cells that are important in the pathogenesis of atherosclerosis are monocytes, i.e. round or oval leukocytes with a kidney-bean-shaped nucleus that mostly originate from hematopoietic stem cells in the bone marrow, after which a part enters the blood circulation and the other part migrates to the spleen for storage (Pittet, Nahrendorf, & Swirski, 2014; Skinner & Johnson, 2017). However, recent studies have suggested that inflammation can lead to the occupation of the spleen by hematopoietic stem cells, giving rise to spleen-derived monocytes (Pittet et al., 2014). Blood circulating monocytes are undifferentiated and possess chemokine and adhesion receptors that arbitrate migration from the blood to the tissues in case of inflammation. They are also capable of invading an infected tissue and thereafter differentiate into tissue resident dendritic cells or macrophages. In addition, monocytes are able to produce inflammatory cytokines, phagocytose pathogens and dying cells (Geissmann et al., 2010).

Macrophages are large leukocytes with a round nucleus. Whereas blood circulating monocytes only survive for 3 days, macrophages and T lymphocytes can live for several months in a tissue (Skinner & Johnson, 2017). The function of macrophages is diverse: recognition of pathogens using pattern recognition receptors, phagocytosis of foreign material and apoptotic cells and the production of inflammatory cytokines (Geissmann et al., 2010; Pittet et al., 2014). Macrophages can be subdivided in 2 phenotypes: M1 and M2 macrophages. The former are classically activated macrophages and contribute the first defense line. In an inflammatory environment, rich in LPS and IFN-γ – derived from Th1 lymphocytes or natural killer cells –, these cells are activated, resulting in a release of pro-inflammatory cytokines, such as interleukin 1β (IL-1β), IL-6, IL-12 and IFN-γ, and chemokines, such as MCP-1. Moreover, M1 macrophages exert a high phagocytic activity. In response to Th2-lymphocyte-secreted anti-inflammatory cytokines, e.g. IL-4, IL-10 and IL-13, macrophages can be polarized towards the M2 phenotype. M2 macrophages are involved in a variety of functions, such as the production of anti-inflammatory cytokines, e.g. IL-4 and IL-10 and a regulatory activity towards wound healing, tumour progression, angiogenesis, tissue remodelling and allergic reactions (Atri, Guerfali, & Laouini, 2018; S. Chen, Yang, Wei, & Wei, 2019; Martinez & Gordon, 2014).

Platelets or thrombocytes have no nucleus and are the smallest of all blood cells (George, 2000). They are blood components derived from megakaryocytes in the bone marrow. In normal conditions, endothelial cells excrete thrombomodulin and prostacyclins which prevent platelet aggregation (Saad & Schoenberger, 2019). The main function of platelets is to arrest bleeding by clumping together to form a hemostatic plug (Johnson, Van Horn, Pederson, & Marr, 1966). Furthermore, they have an essential role in the sustainment of a pro-inflammatory environment through excretion of pro-inflammatory mediators such as TNF-α and recruitment of inflammatory cells such as neutrophils and lymphocytes (Lievens & von Hundelshausen, 2011).

1.1.3.2 Cells of the adaptive immune system

T lymphocytes or T cells, leukocytes with a spherical nucleus, are part of the adaptive immune system (Skinner & Johnson, 2017). They originate from precursor cells in the bone marrow, migrate to the thymus where they differentiate into different types of T cells and continue their maturation in the spleen, lymph nodes or mucosal associated lymphoid tissue. Cells of the innate immune system e.g. dendritic cells recognize pathogen-associated molecular patterns (PAMPs) and subsequently present the specific antigens to the T cells in the lymph nodes. After antigen presentation, naive T cells, i.e. cells that have never encountered an antigen before, will differentiate into CD4 T cells or CD8 T cells. CD4 T cells are defined as T helper (Th) cells, while CD8 T cells are cytotoxic T cells which produce cytolytic proteins which enables them to kill pathogen-infected cells (Salam et al., 2013).

1.1.4 The involvement of the immune system in the pathogenesis of atherosclerosis

Upon endothelial dysfunction, the expression of cytokines and chemokines, such as IL-1, IL-6, MCP-1, macrophage inflammatory protein-1 (MIP-1) and TNF-α, is increased in endothelial cells, which will activate blood circulating monocytes and induce attraction of the monocytes to the vascular wall, as illustrated in Figure 4 (Bobryshev, 2006). L-selectins are expressed on the cell membrane of the monocytes, whereas P- and E-selectins are expressed on endothelial cells. All three selectins bind to sialylated and fucosylated carbohydrates that are found on both cell types (Hidalgo, Peired, Wild, Vestweber, & Frenette, 2007). As monocytes roll along the vascular wall, interaction of selectins with their corresponding ligands slow the rolling movement. However, a second, stronger attachment is required for the arrest of monocytes. This is achieved by the binding of β1- and β2-integrins – expressed by monocytes – with VCAM-1 and ICAM-1, respectively, on endothelial cells (Bobryshev, 2006; Hubbard & Rothlein, 2000; D.-H. Kong, Kim, Kim, Jang, & Lee, 2018). Hereafter, the monocytes escape the blood vessel by a process called diapedesis. Platelet endothelial cell adhesion molecules 1 (PECAM-1) are found on both endothelial cells as monocytes and bind homophilically (Sullivan & Muller, 2014). Junctional adhesion molecules (JAM) are localized at the endothelial cell-cell junctions and their ligands among which integrin α4β1 or very late antigen 4 (VLA-4) are expressed on monocytes (Gerhardt & Ley, 2015). These interaction mechanisms mediate the extravasation of monocytes, wherein the latter form pseudopodia to transmigrate through the endothelium in a paracellular or transcellular way (Sage & Carman, 2009).

Once arrived in the intima, monocytes differentiate into macrophages due to the micro-environmental presence of i.a. macrophage colony-stimulating factor (M-CSF) which is secreted by endothelial cells and vascular smooth muscle cells in response to stimuli such as LPS and TNF-α (Clinton et al., 1992). Monocyte-derived macrophages recognize oxLDL as a damage-associated molecular pattern (DAMP[2]). OxLDL are ligands for the scavengers receptors on the surface of the macrophages and binding results in the internalisation of the oxLDL-receptor complex. Accumulation of oxLDL leads to the formation of foam cells, i.e. lipid-loaded macrophages rich in cholesteryl esters. In contrast to native LDL receptors, scavenger receptors are not downregulated by accumulation of cholesterol in the macrophages. One consequence is the unlimited uptake of lipids until the cells die. Reverse cholesterol transport is supported by high levels of HDL and apoA-I, i.e. a major component of the HDL complex, and causes the shrinkage of macrophage cells, making it an anti-atherogenic process (Falk, 2006). Foam cells and macrophages produce inflammatory cytokines among which IL-1, IL-6, TNF-α and IFN-γ. These cytokines promote inflammation and contribute to the acceleration of the disease development (Marchio et al., 2019). Besides the production of inflammatory cytokines, macrophages produce matrix metalloproteinases. These enzymes degrade the extracellular matrix, causing a destabilizing and thrombogenic effect (Falk, 2006). Hypoxia, i.e. a state of reduced oxygen levels that may occur during plaque-formation, also enhances the production of matrix metalloproteinases. Furthermore, it promotes

[2] DAMPs or alarmins are host danger biomolecules released by damaged or necrotic cells in order to promote an inflammatory response (Roh & Sohn, 2018).

monocyte and macrophage survival and the uptake of oxLDL. It's also associated with adenosine triphosphate (ATP)-depletion leading to cell death which contributes to the growth of the necrotic core, thereby enlarging the plaque which in turn amplifies hypoxia (Parma et al., 2017).

T cells are also involved in the pathogenesis of atherosclerosis, but in contrast to macrophages, they are not part of the first defense line. Whereas macrophages are prominent in early lesions, lymphocytes become the dominant immune response mediators for the enhancement of inflammation in mature lesions. T cells belong to the adaptive immune system and antigen-presentation is required for their activation. OxLDL is thought to emerge as an autoantigen for the activation of T cells (Shi, 2010; Tse, Tse, Sidney, Sette, & Ley, 2013). It is observed that in (the environment of) atherosclerotic plaques, Th1, Th17 and Th2 cells increase in number, while regulatory T (Treg) cells decrease. The latter produce anti-inflammatory cytokines IL-10 and TGF-β and are therefore atheroprotective (Marchio et al., 2019).

Platelets can adhere to activated endothelium due to P-selectin and integrin binding. They promote inflammation by producing pro-inflammatory cytokines such as IL-1β and they attract leukocytes by releasing chemokines (Badimon, Padró, & Vilahur, 2012; Nording, Seizer, & Langer, 2015). Furthermore, platelets are able to regulate angiogenesis which is a crucial factor in plaque growth and instability (Camaré, Pucelle, Nègre-Salvayre, & Salvayre, 2017; Kisucka et al., 2006; Nording et al., 2015). That way, they can contribute to plaque rupture, followed by thrombus formation. Exposure of the content of the atherosclerotic plaque – e.g. extracellular matrix components like collagen, vWF and macrophage-derived tissue factor – to the flowing blood causes platelet activation and aggregation (Badimon et al., 2012; Fuster et al., 1990)

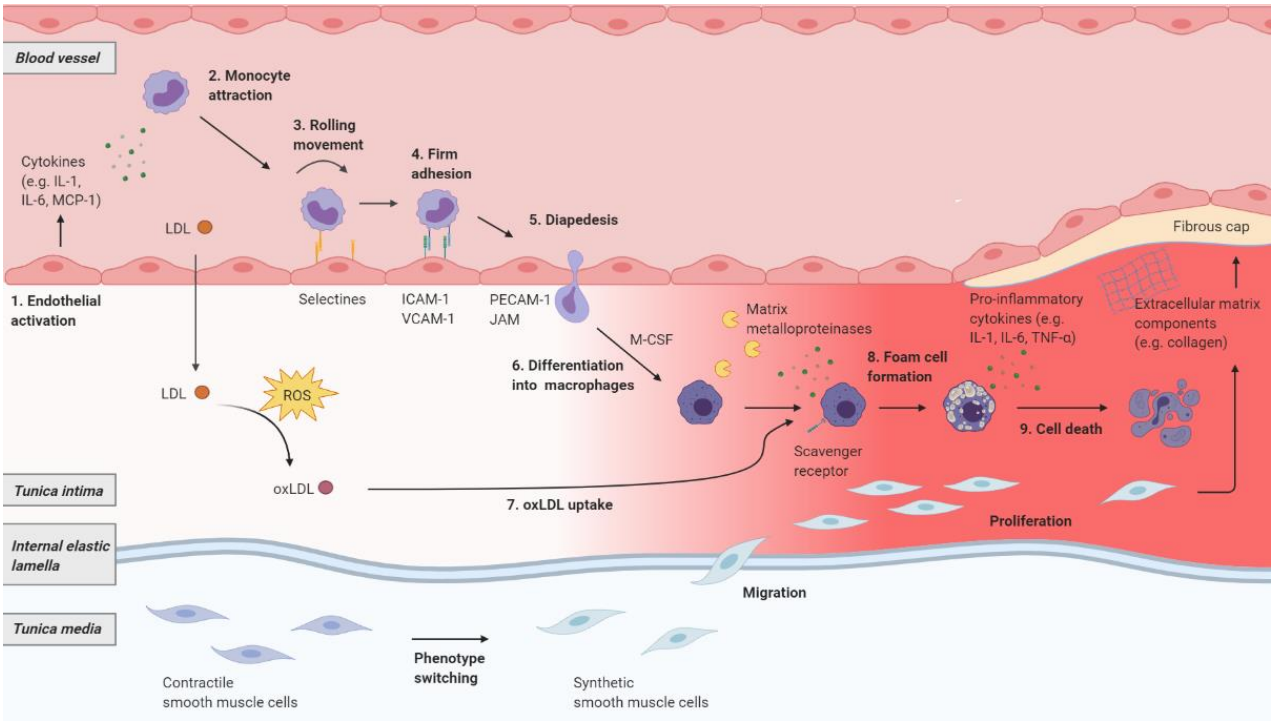


Figure 4. An overview of the pathogenesis of atherosclerosis

1.1.5 Psychological stress as a risk factor for atherosclerosis

As mentioned in section 1.1.2 and illustrated in Figure 3, risk factors for atherosclerosis include obesity, diabetes, hypertension, but also behavioural parameters such as smoking and physical activity. Besides these traditional risk factors, it is reported that psychological factors such as anxiety and depression can play a role in the development of atherosclerosis. Psychological stress can be subdivided in acute stress as a result of a traumatic or surprising event and chronic stress such as job-strain, poor socio-economic status and social isolation. Acute psychological stressors such as anger and fear after e.g. a terroristic attack or an earthquake is shown to be associated with an increase in acute cardiovascular syndromes such as myocardial infection and stroke (Lagraauw, Kuiper, & Bot, 2015). Furthermore, a correlation between chronic stress and increased risk of coronary heart disease is demonstrated in several epidemiological studies (Lagraauw et al., 2015; Steptoe & Kivimaki, 2012).

Stress activates the sympathetic nervous system, hypothalamus-pituitary-adrenal axis and renin-angiotensin-aldosterone system leading to the release of the main stress hormones, catecholamines e.g. adrenaline and glucocorticoids e.g. cortisol. After cessation of the acute stressor, circulating levels of catecholamine and glucocorticoids return to baseline. However, chronic stress can cause maladaptation in the sympathetic nervous system and hypothalamus-pituitary-adrenal axis resulting in lasting increased circulating and local catecholamine and glucocorticoid concentrations (Gu, Tang, & Yang, 2012; Lagraauw et al., 2015). It is evinced that (psychological) stress facilitates chronic low-grade inflammation, thereby contributing to the progression of atherosclerosis (Lagraauw et al., 2015).

1.1.5.1 (Nor)adrenaline

After stressor onset, catecholamines are immediately released and return shortly after stressor termination to baseline levels. Catecholamines are capable of activating different pathways by binding to adrenoreceptors, i.e. G protein-coupled, membrane bound receptors. A positive correlation between increased levels of catecholamines and NF-κB activity is described in literature (Wolf, Rohleder, Bierhaus, Nawroth, & Kirschbaum, 2009). NF-κB is an important regulator of several genes including genes of pro-inflammatory cytokines and adhesion molecules. Interestingly, there is a discrepancy in the reported effect of adrenaline on NF-κB activity. For example, Jin et al. (2011) observed an increased nuclear translocation of NF-κB and subsequent increased adhesion molecule levels in adrenaline-treated endothelial cells. Adrenaline-induced NF-κB activation was also observed in macrophages (Flierl et al., 2009). Other studies reported no or adverse effects (Bierhaus et al., 2003; Zuo, Shi, & Yan, 2016). Adrenaline is a strong stimulator of β-adrenoreceptors which are coupled to G_s-proteins that activate adenylate cyclase. This results in increased cyclic adenosine monophosphate (cAMP) levels, followed by activation of cAMP-dependent protein kinase A (PKA). Described evidences regarding the effect of cAMP/PKA on NF-κB activity are contradictory, which may explain the indistinctness about the adrenaline effect (Gerlo et al., 2011; Takahashi, Tetsuka, Uranishi, & Okamoto, 2002). It is evinced that noradrenaline and adrenaline enhances proliferation of *in vitro* endothelial cells, suggesting that catecholamines trigger endothelial activation which is an important event in the onset of atherosclerosis (Hauss, Bauch, & Schulte, 1990). However, Toda and Nakanishi-Toda (2011) described eNOS activation by (nor)adrenaline, suggesting that catecholamines are not involved in endothelial function

impairment. Although an indirect effect is proposed, since (nor)adrenaline prepares the body for a fight-or-flight response by increasing the heart rate and blood pressure, thereby leading to hypertension, a risk factor for atherosclerosis.

1.1.5.2 Cortisol

Cortisol compensates energy loss during acute stress by mobilizing the body's energy store (Lagraauw et al., 2015). In contrast to catecholamines, it is released after approximately 10 minutes after stressor onset and returns an hour after cessation to baseline levels. Cortisol is known to attenuate immune processes in order to prevent an overreaction of the immune system caused by catecholamines. After binding of cortisol with glucocorticoid receptor in the cell cytoplasm, the complex is translocated to the nucleus where it can activate or inhibit the transcription of several genes, e.g. IκB-α, an inhibitory protein of NF-κB (Wolf et al., 2009). Chronically elevated cortisol levels can lead to glucocorticoid receptor resistance, provoking failure of inflammatory response down-regulation (Cohen et al., 2012). Some studies suggested the role of the NO system in cortisol-induced hypertension (Kelly, Mangos, Williamson, & Whitworth, 1998; Kelly, Tam, Williamson, Lawson, & Whitworth, 1998). The influence of glucocorticoids on endothelial dysfunction through eNOS downregulation is described by Toda and Nakanishi-Toda (2011). NO depletion can be enhanced by overproduction of ROS caused by glucocorticoid excess (Iuchi et al., 2003).

1.2 Introduction to quercetin

Endothelial dysfunction and atherosclerosis are characterized by oxidative stress and inflammation. In order to attempt to prevent atherosclerosis development, the oxidative and pro-inflammatory pathways should be targeted. Some natural compounds such as polyphenols are reported to exert inhibiting effects on these pathways (Santhakumar, Battino, & Alvarez-Suarez, 2018). In this thesis, the focus is on quercetin and its potential beneficial effects. However, when investigating bioactivity, bioavailability should be taken into account as well. As an introduction to the absorption and metabolism of quercetin (section 1.2.3), the structure of the intestinal wall is briefly described in section 1.2.1.

Quercetin is a yellow plant pigment that is found in a variety of plant-based food products, more specifically in vegetables (e.g. onion, lettuce, broccoli), fruit (e.g. apple), beverages (e.g. tea, red wine) and dark chocolate (Aherne & O'Brien, 2002; Magrone, Russo, & Jirillo, 2017). Due to the presence of phenol rings in its chemical structure, as illustrated in Figure 5, quercetin belongs to the polyphenol family and can be classified in the flavonoid[3] subfamily and further subclassified in the flavonol group. It is the most abundant flavonoid and known for its anti-oxidant, anti-inflammatory and anti-hypercholesterolemic properties and its protective effects against several disorders among which obesity, hypertension and atherosclerosis (Anand David, Arulmoli, & Parasuraman, 2016). Quercetin is an aglycone that can be transformed into a number of flavonoid glycosides[4] via the replacement of one or more hydroxyl groups by a sugar molecule. It is mainly found in plants as a glycoside derivative, because storage in the plant vacuoles requires an increased hydrophilicity – and thus an increased polarity – of the molecule which can be achieved by glycosylation (Aherne and O'Brien, 2002).

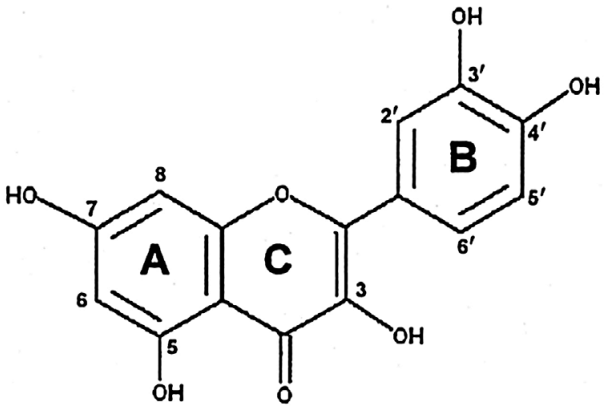


Figure 5. The chemical structure of quercetin (Bustos et al., 2016)

[3] Flavonoids are part of the polyphenol family and are subdivided into 6 classes according to chemical structure: flavonols, flavanols, flavones, flavanones, isoflavones and anthocyanidins.
[4] A glycoside consists of a sugar group (glycone) bound to a non-sugar group (aglycone). Glycosides can be subclassified according to the glycone: e.g. glucosides if the glycone is a glucose, fructosides if the glycone is a fructose, glucuronide if the glycone is a glucuronic acid, etc.

1.2.1 Structure of the intestinal wall

The gastrointestinal tract consists of the mouth, pharynx, esophagus, stomach, small intestine including duodenum, jejunum and ileum, large intestine and anus. The small and large intestine play a key role in the absorption and metabolism of the flavonoid quercetin, which is described in section 1.2.3, therefore, the structure of the intestinal wall is briefly described in the following section and illustrated in Figure 6. The intestinal wall consists of four layers: the mucosa, the submucosa, the muscularis propria and the serosa.

The innermost layer, the mucosa, is made up of 3 layers itself. An epithelial monolayer is in contact with the lumen and is supported by a basement membrane. The underlying lamina propria contains lymph nodes, lacteals and connective tissue. The third layer of the mucosa is the muscular mucosa which is formed by a sheet of smooth muscle cells. In the small intestine, the absorptive function is maximized. The mucosa enlarges its surface area in the small intestine by circular folds, villi, i.e. protrusions that cover intestinal folds, and microvilli, i.e. protrusions on the apical side of the intestinal cells that form a brush border (Collins & Badireddy, 2020). The villi are occupied by differentiated epithelial cells such as enterocytes – i.e. columnar absorptive cells –, enteroendocrine – i.e. hormone and peptide secreting cells – and mucus secreting Goblet cells. Between the villi, small invaginations, called crypts of Lieberkühn, are located. These contain another type of differentiated epithelial cells, namely Paneth cells that secrete antimicrobial agents, and stem cells that assure the continuous renewal of the enterocytes (Rao & Wang, 2010). In contrast to the small intestine, the large intestinal epithelium exists of simple columnar absorptive epithelial cells and there are no circular folds or villi present. However, the apical side of colonocytes contains microvilli and along the large intestinal wall, saccules are formed. These are called haustra and are separated by semilunar folds. In the crypts, where stem cells are located, Paneth cells are absent (Jorge & Habr-Gama, 2007). In addition, the enteroendocrine cell population is less diverse and Goblet cells are more prominent in the large intestine, compared to the small intestine. The latter cells provide a thick mucus layer containing antimicrobial molecules (Gunawardene, Corfe, & Staton, 2011; S. Kong, Zhang, & Zhang, 2018; Okumura & Takeda, 2017).

The submucosa consists mainly of connective tissue with embedded blood vessels, lymphatics and nerves. The muscularis propria comprises two layers that are responsible for the motility of the intestine: the inner circular layer and the outer longitudinal layer (Azzouz & Sharma, 2020).

The outermost layer, the serosa, contains connective tissue and mesothelium, i.e. squamous epithelium in order to reduce friction. In case the latter is missing, the outermost layer is called the adventitia (Rao & Wang, 2010).

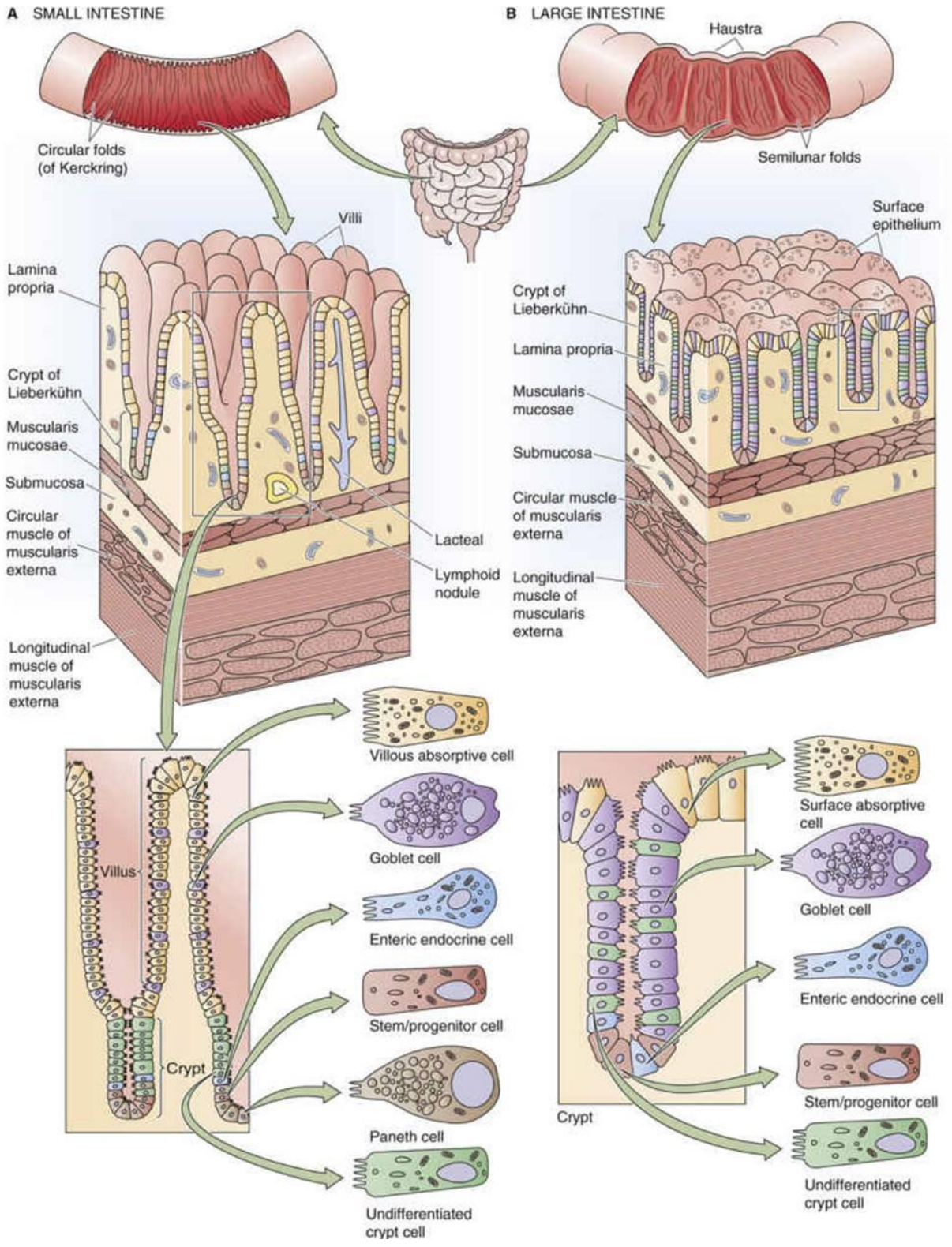


Figure 6. Structure of the small intestinal wall (A) and large intestinal wall (B) (Boron & Boupaep, 2016)

1.2.2 Bioavailability of quercetin

Since flavonols are not produced in the human body, they can only be derived from food or supplements (Anand David et al., 2016). The average daily flavonoid intake is estimated between 50 and 800 mg of which quercetin intake amounts about 75%, dependent on the consumption of quercetin-rich food like fruits, vegetables and tea (Y. Li et al., 2016). Although daily quercetin (glycosides) intake is relatively high compared to other flavonoids, its rather low *in vivo* bioavailability should be taken into account.

A clinical study with 9 healthy subjects who ingested supplements of 154 mg quercetin-4'-glucoside or 151 mg quercetin-3-glucosides, suggests that the bioavailability of both quercetin glucosides are similar – with an average $AUC_{0\rightarrow72h}$ of 5.8 h*µg/mL (19.1 h*µM) and 5.3 h*µg/mL (17.5 h*µM), respectively –, so the impact of the position of the glucose moiety is negligible (Olthof, Hollman, Vree, & Katan, 2000). However, it is evinced that quercetin homoglucosides are absorbed more rapidly than quercetin aglycone and other quercetin glycosides in a rat model (Morand, Manach, Crespy, & Remesy, 2000). A possible elucidation for this is given in the following paragraph where the absorption mechanism of quercetin (derivatives) is explained in more detail.

Bioavailability of quercetin (glycosides) is relatively low and differs according to the food matrix, the sugar moiety of the glycoside and the simultaneous uptake of dietary components e.g. fibers and fat (Y. Li et al., 2016; Trakooncharoenvit, Tanaka, Mizuta, Hira, & Hara, 2019). For example, in a clinical trial with 9 participants it is demonstrated that dietary fat facilitates quercetin aglycone absorption via passive diffusion in the small intestine by enhancement of micelle incorporation and thereby improves its bioavailability (Y. Guo et al., 2013). The influence of food matrix and sugar moiety on bioavailability is demonstrated by Graefe et al. (2001). A study with 12 healthy volunteers was performed in order to compare the bioavailability of quercetin in onion and buckwheat tea powder – which contain high amounts of quercetin-4'-glucoside and rutin, respectively –, pure quercetin-4'-glucoside and rutin. The administered quantity of onion and quercetin-4'-glucoside is equivalent to 100 mg quercetin and the quantity of buckwheat tea powder and rutin is equivalent to 200 mg quercetin. As displayed in Table 1, the bioavailability of quercetin in onion and quercetin-4'-glucoside is significantly higher than rutin and buckwheat tea. The bioavailability of quercetin in onion and buckwheat tea is higher than pure quercetin-4'-glucoside and rutin, respectively, which affirms the importance of the food matrix. Furthermore, it can be concluded that quercetin-4'-glucoside is faster absorbed than rutin, which is in line with the aforementioned statement of Morand et al. (2000).

Table 1. Bioavailability of quercetin derivatives in different food matrices (Graefe et al., 2001)

Quercetin derivative	**Quercetin equivalent (mg)**	**Food matrix**	**$AUC_{0\rightarrow 24h}$ (h*µg/mL)**	**C_{max} (µg/mL)**	**t_{max} (h)**
Quercetin-4'-glucoside	100	Onion	9.7 ± 6.9	2.31 ± 1.46	0.68 ± 0.22
Rutin	200	Buckwheat tea	3.8 ± 3.9	0.64 ± 0.67	4.32 ± 1.83
Quercetin-4'-glucoside	100	/	8.4 ± 9.1	2.12 ± 1.63	0.70 ± 0.3
Rutin	200	/	2.5 ± 2.2	0.32 ± 0.34	6.98 ± 2.94

Another study with 16 healthy volunteers aimed to determine the bioavailability of quercetin aglycone and rutin. The average $AUC_{0\rightarrow 32h}$, C_{max} and t_{max} after intake of 50 mg quercetin aglycone and 100 mg rutin amounts 1.3 h*µg/mL, 0.0861 µg/mL, 4.9 h and 1.2 h*µg/mL, 0.0899 µg/mL, 7.5 h, respectively. Especially after rutin ingestion, a great inter-individual variability was observed, this can be due to the dependence on microbiota for the metabolism of rutin (which will be discussed in section 1.2.3). The bioavailability of quercetin from rutin seemed to be higher in women compared to men and the highest in women taking oral contraceptives (Erlund et al., 2000). Inter-individual variability was also noticed in other recent studies, i.a. by Almeida et al. (2018) who reviewed 55 intervention trials concerning quercetin bioavailability (Almeida et al., 2018; Erlund, 2004; Graefe et al., 2001). Erlund (2004) suggests on the one hand physiological factors – e.g. body weight, gender, body composition, gastrointestinal microbiota and gastric motility – and on the other hand molecular factors – e.g. synthesis and activity of transporters and metabolic enzymes – to be a cause of this variation. Health status is also brought up by Almeida et al. (2018) as a potential factor affecting inter-individual variability in quercetin bioavailability.

1.2.3 Absorption and metabolism of quercetin

Crespy et al. (2002) demonstrated that quercetin aglycones may be absorbed in the stomach, however, this is not the case for quercetin glycosides. Due to low pH, quercetin is protonated, enabling passage through the stomach wall, although absorption in the stomach is rather limited (Rich, Buchweitz, Winterbone, Kroon, & Wilde, 2017). Most of the ingested quercetin (glycosides) are absorbed near the intestinal wall. Due to the higher hydrophilicity of quercetin glycosides, compared to the aglycone, passage through the enterocyte cell membrane is hindered. Quercetin glycosides can be first hydrolysed to aglycones by lactase-phlorizin hydrolase enzyme (LPH) – located in the brush border – before entering the enterocyte. Another option is the uptake in enterocytes via carrier-mediated transport (by the sodium-dependant glucose transporter 1 (SGLT1)) after which they are hydrolysed intracellular by cytosolic β-glucosidase. Studies also suggested that organic anion transporting polypeptide (OATP) and bilitranslocase may be involved in this carrier-mediated transport. The transport of quercetin-4'-β-glucoside and quercetin-3-glucoside by SGLT1 has already been demonstrated (Walgren, Lin, Kinne,

& Walle, 2000; Wolffram, Blöck, & Ader, 2002). This could be an explanation for the faster absorption of quercetin glucosides compared to quercetin aglycones.

Due to the lipophilic nature of quercetin aglycone, passive diffusion through the phospholipid bilayer of enterocytes is facilitated (Murota & Terao, 2003). However, Chabane et al. (2009) reported that pH-dependent transport of quercetin aglycone via OATPs – i.e. apical membrane transporters that belong to the solute carrier (SLC) family – in Caco-2 cells is also possible at low pH.

After entering the enterocytes, quercetin is either directly absorbed into the hepatic portal vein or undergoes metabolism in two phases: phase I includes oxidation and O-demethylation and phase II consists of methylation, sulfation or glucuronidation. Cytochrome P450 enzymes that are membrane-bound within the endoplasmic reticulum (ER) are mostly involved in phase I metabolism (Phang-Lyn & Llerena, 2020). Phase II metabolism is predominant and involves enzymes such as cytoplasmic soluble catechol-O-methyltransferases (COMTs), cytosolic phenol sulfotransferases and ER-membrane-bound UDP-glucuronyl transferases (UGTs) (Jancova, Anzenbacher, & Anzenbacherova, 2010). The resulting quercetin conjugates are absorbed into the bloodstream via passive diffusion or basolateral ATP binding cassette (ABC) transporters (Dabeek & Marra, 2019; Graf et al., 2006). ABC transporters feature ATPases enabling them to hydrolyse ATP and use the harnessed energy for active efflux transport of molecules. The ABC transporter family includes multidrug resistant proteins (MRPs), breast cancer resistant protein (BCRP) and P-glycoprotein (P-gp) (Speer et al., 2019). *In vivo* studies in a rat showed that both MRP3 as MRP4 are basolaterally located, facilitating the active absorption of quercetin (metabolites) in the blood stream (Proctor et al., 2016). In contrast, P-gp, MRP2 and BCRP are located at the apical membrane of enterocytes, resulting in an efflux of flavonoids back to the intestinal lumen (Almeida et al., 2018). According to Chabane et al. (2009), quercetin is not a substrate of P-gp in Caco-2 cells, but it is a substrate for MRP2 and BCRP. This active efflux to the lumen, together with limited passive diffusion, results in a low bioavailability of quercetin (glycosides).

Moreover, glycosides containing a rhamnose unit, such as rutin (quercetin-3-rutinoside), are not absorbed in the small intestine (Morand et al., 2000). Deglycolysation of these quercetin glycosides is mediated in the colon by bacteria that contain both β-glucosidases and α-rhamnosidases such as *Bifidobacterium dentium*. This can explain the great inter-individual variability regarding rutin bioavailability, as observed by Erlund et al. (2000). The quercetin backbone structure can be further broken down into phenolics by bacterial fission of the C-ring (Braune & Blaut, 2016). The resulting compounds can be either absorbed into the bloodstream or excreted in the faeces (Dabeek & Marra, 2019).

When absorbed in the bloodstream, quercetin (metabolites) may bind to albumin and is transported to the liver via the portal vein (Manach et al., 1996). Once arrived in the liver, quercetin (conjugates) are further metabolized by phase I and II reactions (Iyer & Sinz, 1999). Quercetin glucuronides that enter hepatocytes can be first hydrolysed or directly methylated by COMT. Deglucuronidation of quercetin-3-glucuronides and quercetin-7-glucuronides by intracellular β-glucuronidase was reported by O'Leary et al. (2003). Graf et al. (2006) demonstrated that the vast majority in the liver consists of quercetin

metabolites of which methylated glucuronated quercetin sulfates were most abundant. The latter can be excreted in the bile, subsequently secreted into the small intestine and after deconjugation by microbiota, they may be reabsorbed (Scalbert & Williamson, 2000; Shimoi, Yoshizumi, Kido, Usui, & Yumoto, 2003). Quercetin (metabolites) can also be transported from the liver to other body tissues such as the brain and lungs or to the kidneys from where they will be excreted in the urine (Dabeek & Marra, 2019). Blood circulating quercetin can exit the blood vessel by crossing the endothelial wall. Uptake in endothelial cells occurs via bilitranslocase, i.e. a bidirectional transporter of both bilirubin and some flavonoids such as quercetin (Cvorovic, Ziberna, Fornasaro, Tramer, & Passamonti, 2018; Maestro et al., 2010). Cvorovic et al. (2018) suggest that vascular endothelial cells also possess other transporters through which flavonoid transport can take place, such as BCRP, OATPs and MRPs. In literature, not much has been written about the metabolism of flavonoids in the endothelium yet. Nevertheless, the presence of metabolizing enzymes such as cytochrome P450, COMTs, UGTs and sulfotransferases (SULTs) in vascular endothelial cells is already confirmed, but their contribution to flavonoid metabolism remains unclear (Gonzales et al., 2015; Hoebel, Steyrer, & Graier, 1998; Tu, Delahunty, Ding, Luscinskas, & Tedder, 1999).

1.3 The beneficial effects of quercetin on the pathogenesis of atherosclerosis

Quercetin is reported to provoke several beneficial effects, such as anti-inflammatory, anti-oxidative, anti-thrombotic and vasodilating effects, and is therefore known to counteract certain diseases such as atherosclerosis and its associated vascular diseases (Salvamani, Gunasekaran, Shaharuddin, Ahmad, & Shukor, 2014). In this section, epidemiological and clinical evidence concerning the beneficial potential of flavonoids towards atherosclerosis will be discussed.

Several prospective cohort studies concerning the association of flavonoid consumption and cardiovascular diseases show an inverse association, whereas others did not observe an association. For example, in a study by Rimm et al. (1996) with 34 789 male health professionals without prevalent cardiovascular disease, including healthy people, hypertensives, smokers, diabetics, etc., no significant inverse association between flavonoid dietary intake and coronary heart disease was observed. However, in a similar study with 4814 men with prevalent coronary heart disease, high flavonoid intake reduced risk of death from coronary heart disease, although not significant (Rimm et al., 1996). This displays the importance of health status on flavonoid responsiveness. A recent prospective cohort study by Dalgaard et al. (2019) with 53 552 participants without any prevalent atherosclerotic cardiovascular diseases found an inverse association between flavonoid intake and risk of atherosclerotic cardiovascular disease. Interestingly, a stronger association was found in the smoking, at-risk subpopulation (Dalgaard et al., 2019). In a prospective cohort study with 34 492 postmenopausal women with no history of prior heart disease/attack, a high dietary quercetin intake was observed to be correlated with a decreased risk of coronary heart disease death (Yochum, Kushi, Meyer, & Folsom, 1999). In another study with 10 054 participants (including healthy people, hypertensives, smokers,

diabetics, etc.), a high dietary intake of quercetin tended to be associated with a lower ischemic heart disease mortality, cancer incidence, risk of type 2 diabetes and asthma incidence (Knekt et al., 2002).

Peterson et al. (2012) summarized the findings of 20 publications from 12 prospective cohort studies concerning the correlation between flavonoid intake and cardiovascular disease incidence/mortality in Europe and the U.S.A. The main conclusions were that from the 8 cohorts on coronary heart disease mortality, 4 observed an inverse association with flavonoid intake – with the strongest association for the flavonol class. Moreover, in 3 out of 7 cohorts, flavonol and flavanone consumption was inversely correlated with stroke incidence (Peterson et al., 2012). Due to the variability of the study designs including population characteristics (e.g. health status), comparison among the different cohorts was complicated. As a result, no subpopulation analysis was performed in the latter study.

To better understand the influence of flavonoids on cardiovascular health, many clinical trials were performed. Egert et al. (2009) concluded from a study with 93 obese subjects that quercetin supplementation decreased oxLDL concentrations in plasma and systolic blood pressure of overweight people with a high risk of cardiovascular diseases. The decrease in blood pressure is also observed in hypertensive patients after quercetin supplementation (Edwards et al., 2007). Brull et al. (2015) performed a study with 68 overweight-to-obese subjects with (pre-)hypertension who were supplemented for 6 weeks with 162 mg/day quercetin in the form of onion skin extract or a placebo. In the subgroup of 31 hypertensive patients, a significant decrease in systolic blood pressure compared to the placebo group was observed. Furthermore, soluble ICAM-1 (sICAM-1) level was significantly reduced after quercetin supplementation, but there was no significant difference with the placebo group. Quercetin did not seem to affect other endothelial dysfunction biomarkers (e.g. sVCAM-1, sE-selectin) (Brull et al., 2015). A significant decline in sE-selectin and IL-1β concentrations by quercetin compared to placebo was observed in a study with 35 non-smoking (pre)hypertensive patients who were given daily a placebo or 160 mg quercetin-3-glucoside for 4 weeks. No significant change in other endothelial dysfunction biomarkers (sVCAM-1, sICAM-1, vWF, MCP-1) and inflammatory mediators (IL-6, IL-8, TNF-α) was noticed (Dower et al., 2015). In a clinical trial with 72 women who suffered diabetes type II, a placebo or 500 mg quercetin was daily given to each participant for 10 weeks. Systolic blood pressure was significantly reduced by quercetin intake compared to placebo. Serum concentrations of TNF-α and IL-6 decreased significantly after quercetin supplementation, however no significant difference with the placebo group was observed (Zahedi, Ghiasvand, Feizi, Asgari, & Darvish, 2013). In a study by K. H. Lee et al. (2011), 92 non-diseased male smokers were given 100 mg quercetin supplementation or a placebo for 10 weeks. Both systolic and diastolic blood pressure and total cholesterol and LDL levels declined significantly in the quercetin group, although differences with the placebo group were not significant for all of these variables. No significant effect on the concentrations of sVCAM-1 and IL-6 was detected.

In the aforementioned studies by Egert et al. (2009), Edwards et al. (2007), Brull et al. (2015), Dower et al. (2015), Zahedi et al. (2013) and K. H. Lee et al. (2011), a positive effect of quercetin towards blood pressure and/or endothelial dysfunction and inflammation was observed in the at-risk subpopulations. Unfortunately, no control groups with healthy subjects were included in these study designs.

Larson et al. (2012) did use a control group with 5 normotensive men, besides the group of interest with 12 stage 1 hypertensive men. A placebo or 1095 mg quercetin was ingested by the participants every day for 4 weeks. Interestingly, a significant drop in systolic, diastolic and mean arterial pressure by quercetin compared to placebo was only observed in the hypertensive subjects. No effect was seen in the normotensive group. Despite the increase of plasma quercetin concentration, supplementation with quercetin did not significantly alter risk factors of cardiovascular diseases and thrombosis, such as blood pressure, oxLDL levels, inflammation and platelet aggregation in studies with healthy participants (Conquer, Maiani, Azzini, Raguzzini, & Holub, 1998; Egert et al., 2008; Knab et al., 2011). These studies are in line with the conclusion of Almeida et al. (2018) regarding the impact of health status on inter-individual variability in quercetin bioavailability.

It should be noted that in none of the aforementioned clinical trial studies quercetin metabolites were identified in the blood samples. While Dower et al. (2015), Zahedi et al. (2013) and K. H. Lee et al. (2011) did not even measure quercetin concentrations in the blood, Egert et al. (2009), Edwards et al. (2007), Brull et al. (2015), Larson et al. (2012), Conquer et al. (1998), Egert et al. (2008) and Knab et al. (2011) first hydrolysed the samples before analysing the total quercetin concentrations. Consequently, it is not known which quercetin metabolites provoke the observed effects. In order to clarify this, it is recommended to identify and quantify the quercetin metabolites present in the blood in future clinical trials.

1.4 *In vitro* models used to investigate flavonoid bioavailability and bioactivity towards endothelial dysfunction and atherosclerosis

The epidemiological and clinical studies of section 1.3 indicate potential beneficial effects of flavonoids such as quercetin on i.a. atherosclerosis, especially for at-risk subpopulations. However, in order to understand the pathways behind these effects, mechanistic research is required. *In vitro* models serve as an interesting tool to unravel cellular pathways and are therefore often used for mechanistic research (Chanput, Mes, & Wichers, 2014). In section 1.1, the importance of endothelial cells and monocytes in the context of atherosclerosis was discussed, while in section 1.2, the role of the intestine in the uptake and metabolism of flavonoids was described. Therefore, in this section, cell lines used as a model for intestinal cells, endothelial cells and monocytes, are discussed.

1.4.1 Cell lines used for *in vitro* model development to investigate flavonoid bioavailability and bioactivity towards endothelial dysfunction and atherosclerosis

1.4.1.1 Caco-2 cells used as model of intestinal epithelial barrier

The Caco-2 cell line was originally established from a human colon adenocarcinoma and is the principle cell line used as *in vitro* model for the human intestine. When Caco-2 cells are reaching confluence in cell culture, they start to differentiate spontaneously, thereby obtaining intestinal columnar epithelial characteristics such as an apical brush border with microvilli. Moreover, they are forming monolayers

with tight junctions between adjacent cells. To improve polarization, Caco-2 cells can be grown on a filter that mimics the steric conditions of the epithelium *in vivo*. Upon differentiation, Caco-2 cells derive functional properties of enterocytes such as the expression of enzymes and transporter molecules (Lea, 2015). However, many enzymes and transporters often have a lower expression in Caco-2 cells than in enterocytes *in vivo*. This was demonstrated for i.a. MRP1, MRP2 and cytochrome P450 3A, i.e. a phase I enzyme (Nakamura et al., 2002). The gene expression profiles of transporter proteins in differentiated Caco-2 cells are most similar to the human ileum, whereas the expression profile of metabolizing enzymes has the highest resemblance with colon tissue (Landowski, Sun, & Amidon, 2005).

Examples of expressed transporter proteins that are associated with flavonoid absorption dynamics are P-glycoprotein, MRP1, MRP2, glucose transporters (GLUTs) and SGLT1 (Landowski et al., 2005). Both MRP2 and SGLT1 are expressed at the apical membranes, but the latter is particularly found intracellular where it presumably serves as a reserve pool (Kipp, Khoursandi, Scharlau, & Kinne, 2003; Q. Yang, Onuki, Nakai, & Sugiyama, 2007). Caco-2 cells express a broad range of enzymes, i.a. hydrolases (e.g. β-glucosidases), peptidases, phosphatases and conjugating enzymes (Meunier, Bourrie, Berger, & Fabre, 1995; Rydzewski, 2008). Brush border enzymes in Caco-2 cells include sucrase isomaltase, dipeptidylpeptidase IV and LPH (Hauri, Sander, & Naim, 1994). Phase II enzymes such as glutathione S-transferase (GST), SULT, N-acetyltransferase, COMT and UGT were detected, however the activity of UGT was rather low (Chung et al., 2018; Lnenickova et al., 2016; Prueksaritanont, Gorham, Hochman, Tran, & Vyas, 1996). As discussed above, LPH, β-glucosidase, COMT, UGT and SULT are found to be important enzymes in the *in vivo* flavonoid metabolism.

Caco-2 cells have stress receptors that can induce a pathway in response to stressors. For example, TNF-α is a ligand for TNF receptor 1 (TNFR1) and 2 (TNFR2) and basolateral stimulation of Caco-2 cells with TNF-α can upregulate the secretion of pro-inflammatory cytokines such as IL-8 and IP-10 (Sonnier, Bailey, Schuster, Lentsch, & Pritts, 2010; Treede et al., 2009). Production of cytokines such as IL-6, IL-8, IL-10 and TNF-α is also shown to be upregulated after bacterial stimulation (Parlesak, Haller, Brinz, Baeuerlein, & Bode, 2004). Sonnier et al. (2010) suggested that TNFR2 is only active on the apical membrane, whereas TNFR1 is active at both sides.
Toll-like receptor 4 (TLR4) – that recognizes LPS – is another stress receptor expressed on Caco-2 cells (Mendoza, Matheus, Iceta, Mesonero, & Alcalde, 2009). Suzuki et al. (2003) reported that Caco-2 cells are relatively hyporesponsive to LPS. IL-8 production as a result of LPS stimulation was only observed at concentrations of 1 µg/mL LPS or greater (Suzuki et al., 2003). NOS plays a key role in the pathogenesis of atherosclerosis and according to Vignoli et al. (2001), it is constitutively expressed at low levels in differentiated Caco-2 cells. Inducible NOS (iNOS) can be activated in response to inflammatory agents such as IFN-γ and phorbol-12-myristate-13-acetate (PMA). There is some controversy in literature about the presence and localisation of adrenergic receptors in Caco-2 cells. While Odore et al. (2003) discussed the expression of both β1- and β2-adrenergic receptors in differentiated Caco-2 cells, Abraham et al. (2004) referred to Caco-2 cells as β2-receptor negative cells. Likewise, a pathway involving α2-adrenergic receptors in proliferating Caco-2 cells was discussed by El Moussawi et al. (2018), whereas the absence of this receptor was suggested in several other studies

(Berlioz et al., 2000; Musch, Arvans, Paris, & Chang, 2009). In case of presence of the adrenergic receptors, the precise localisation in Caco-2 cells is not described in literature so far. However, in colonic cancer HT-29 cells, in Manin-Darby Canine Kidney (MDCK) cells and in enterocytes *in vivo,* α2-adrenergic receptors are almost exclusively located in the basolateral membrane, which makes sense, since its agonists – catecholamines – circulate in the bloodstream (Keefer & Limbird, 1993; Saunders & Limbird, 1999; Valet et al., 1993). The presence of cytosolic glucocorticoid receptors in Caco-2 cells is confirmed by several studies (Raddatz, Toth, Schworer, & Ramadori, 2001; Sunil, Ramadori, & Raddatzc, 2010).

1.4.1.2 EA.hy926 cells used as model for endothelial cells

The EA.hy926 cell line is an immortalized cell line, originally generated by fusion of the human lung carcinoma A549 with human umbilical vein endothelial cells (HUVECs) (Bouïs, Hospers, Meijer, Molema, & Mulder, 2001). HUVECs are primary endothelial cells that are derived from the vein of the umbilical cord (Larrivee & Karsan, 2005). The advantage of permanent cell lines compared to primary cells is the time-saving due to the circumvention of the preparation and passages of the latter (Lidington, Moyes, McCormack, & Rose, 1999).

Just as in human aortic primary endothelial cells, the presence of bilitranslocase is confirmed in EA.hy926 cells, permitting the uptake of flavonoids such as quercetin (Maestro et al., 2010). The expression of P-glycoprotein, MRPs and OATPs in EA.hy926 cells is described in literature, however, their role in quercetin transport is not mentioned so far (Ajithkumar, Vinitha, Binil Raj, & Kartha, 2016; Ansari & Brown, 2006). Metabolizing enzymes that are found in vascular endothelial cells *in vivo*, such as cytochrome P450, SULTS, COMTs and UGTs, are deduced to be present as well in EA.hy926 cells (Hoebel et al., 1998; X. Li et al., 2001; Toro-Funes et al., 2015).

Unger et al. (2002) demonstrated the upregulation of MCP-1 upon LPS stimulation in EA.hy926. In addition, LPS also upregulates the expression of its receptor, TLR4, in EA.hy926 (Liu et al., 2015). Cultured HUVEC often possess a diminished number of caveolae. This is not the case for the EA.hy926 cell line which displays an extensive caveolar system similar to *in situ* endothelial cells. TNFR1 is localized in these caveolae and binding with TNF-α results in internalisation of the complex (D'Alessio, Al-Lamki, Bradley, & Pober, 2005).

EA.hy926 cells constitutively express adhesion molecules (ICAM-1, LFA-3, PECAM-1), receptors (Fas), cytokines (IL-6, MCP-1, GM-CSF, but not IL-8), CD40, vWF and MHC class I (Lidington et al., 1999; Unger et al., 2002). ICAM-1 expression is upregulated in EA.hy926 cells upon TNF-α or IFN-γ stimulation. This effect was not observed with IL-1β and LPS. However, in several other studies an upregulation of ICAM-1 expression by LPS in EA.hy926 cells was observed (Thor et al., 2010; Yuan et al., 2019). Lidington et al. (1999) concluded that, in contrast to HUVEC, VCAM-1 and E-selectin showed no detectable upregulation after administration of TNF-α. However, this is in disagreement with the results of a study of Thornhill et al. (1993). EA.hy926 cells may be less sensitive to the effects of stressors compared to primary HUVEC cells, as demonstrated by Unger et al. (2002), but they are capable of expressing VCAM-1, ICAM-1 and E-selectin in response to TNF-α (Deng et al., 2019). EA.hy926 cells can be used as a model for oxidative modification of LDL to mimic the *in vivo* situation.

The mechanisms of LDL modification were very similar to those of HUVECs (Pech-Amsellem et al., 1996). The cell line is also capable of producing NO due to its possession of eNOS (Mashimo, Ishikawa, Numakura, Kinoshita, & Teramoto, 2013). Cytosolic glucocorticoid receptors are expressed in EA.hy926 cells (Newton et al., 2003). The presence of both α- and β-adrenergic receptors at the apical membrane is perceived on endothelial cells and evidence for β-receptors in EA.hy926 cells is reported in literature as well (Jain, Sahu, & Hanif, 2020; Sorriento, Trimarco, & Iaccarino, 2011).

1.4.1.3 THP-1 cells used as model for human monocytes

The immortalized THP-1 cell line is derived from the peripheral blood of an acute monocytic leukemia patient. It is often used as a model for human monocytes in order to uncover their function in healthy and diseased condition (Bosshart & Heinzelmann, 2016). Qin (2012) reported that this cell line is highly used in studies related to atherosclerosis. An advantage of THP-1 cells in comparison to primary monocytes is the lower variability in cell phenotype due to their homogeneous genetic background. This is essential in studies with e.g. oxLDL that are characterized by a high variability and therefore require a lot of replications. Just as monocytes *in vivo*, THP-1 monocytes are able to differentiate into a macrophage-like phenotype upon addition of e.g. PMA or M-CSF (Chanput et al., 2014). Differentiation can be ascertained by macrophage cell surface markers such as CD11b and CD36 (Zhou et al., 2010). The expression of MRP2, P-gp and BCRP-1 in THP-1 macrophages is described in literature (Matt et al., 2017; Perdomo et al., 2016). The presence of metabolizing enzymes such as cytochrome P450, UGTs and phenol sulfotransferases in the THP-1 cell line is evinced by several researchers (Barbier et al., 2003; H. Huang, Yu, & Wang, 2014; X. Huang et al., 2015). The THP-1 cell line shows a lot of similarities with primary monocytes, however, it also differs in some areas. The production of pro-inflammatory cytokines – e.g. TNF-α, IL-6 and IL-8 – in response to LPS is i.a. smaller. The high expression of the toll-like receptor complex – formed by CD14, MD2 and TLR4 – in primary cells could be a potential cause (Bosshart & Heinzelmann, 2016; Chanput et al., 2014). Besides TLR4, the expression of both TNFR1 and TNFR2 is confirmed in THP-1 cells (Goppelt-Struebe, Reiser, Schneider, & Grell, 1996).

When the stress receptors are activated, monocytes are able to traffic towards the site of inflammation and adhere to it by means of adhesion molecules. Examples of adhesion molecules that are expressed by THP-1 upon LPS stimulation are ICAM-1 and LFA-1 (Hmama, Knutson, Herrera-Velit, Nandan, & Reiner, 1999; Thornton & McDaniel, 2005). L-selectin, PECAM-1 and JAM – which are essential for monocyte invasion in tissues – are expressed at the surface of THP-1 cells as well (Ogasawara et al., 2009; Rui et al., 2007; Theilmeier et al., 1999). The presence of both glucocorticoid, α- and β-adrenergic receptors in THP-1 cells is confirmed in literature (Grisanti, Perez, & Porter, 2011; B. Li, Bai, & Wanh, 2006; Talmadge, Scott, Castelli, Newman-Tarr, & Lee, 1993).

1.4.2 *In vitro* evidence of flavonoid bioavailability and bioactivity towards endothelial dysfunction and atherosclerosis

To better understand the mechanistic pathways behind the beneficial effects of flavonoids on inflammatory diseases, such as atherosclerosis, *in vitro* assays are performed.

To create an inflammatory situation *in vitro*, cells are often stressed with bacterial or host-related stressors, such as LPS or TNF-α, respectively, – which are associated with bacterial infection and chronic inflammation, respectively – that are atherogenic risk factors. Monocultures or co-cultures with cell lines that are mentioned in section 1.4.1 are frequently used as *in vitro* models. In this section, different *in vitro* models that are used in recent research studies are discussed. The focus is on models where the effect of pure quercetin is examined on i.a. LPS- and TNF-α-stressed cells.

1.4.2.1 Monoculture models used to investigate flavonoid bioactivity towards endothelial dysfunction and atherosclerosis

1.4.2.1.1 Intestinal cells

To evaluate the impact of quercetin absorption and transport, studies are often performed with a Caco-2 cell monolayer. The apparent permeability coefficient (Papp) of quercetin (derivatives) was determined from unilateral transport assays – from the apical to basolateral side or vice versa – in a static Transwell® system in several studies (Contreras, Borrás, Herranz Lopez, Micol, & Segura Carretero, 2015; J.-n. Hu, Zou, He, Chen, & Deng, 2016; Lund & Pantuso, 2014; Rastogi & Jana, 2016; Y. Xie et al., 2014; Y. Yang et al., 2014). In these studies, Papp values from the apical to basolateral side range between 2 * 10^{-6} and 10 * 10^{-6} cm/s after applying 5-100 µM quercetin aglycone. J.-n. Hu et al. (2016) concluded that quercetin transport does not involve paracellular pathways. Furthermore, it was demonstrated that efflux proteins, such as P-gp and MRPs, participate in the transport process. Quercetin transport is suggested to involve both active efflux mechanisms as well as passive diffusion, where the latter is more prominent at higher quercetin concentrations. HPLC is a frequently used method to measure cellular quercetin (metabolite) concentration. The effect of LPS and TNF-α on the quercetin accumulation in Caco-2 cells was demonstrated using spectrofluorometry, flow cytometry and confocal microscopy by Vissenaekens et al. (2019). Proliferating Caco-2 cells stressed with LPS, but not TNF-α, were shown to increase quercetin accumulation.

In a study of Jiang et al. (2019), a Caco-2 cell monolayer was pre-treated with red-osier dogwood extracts – that contains quercetin derivatives –, followed by treatment with TNF-α. Whereas TNF-α alone increased the expression of IL-8, IL-6, ICAM-1, VCAM-1 and TNF-α, red-osier dogwood extract counteracted this effect. By means of an enzyme-linked immunosorbent assay (ELISA), Han et al. (2016) demonstrated that quercetin dose-dependently decreased the expression levels of TNF-α and IL-6 in Caco-2 cells.

1.4.2.1.2 Endothelial cells

Monocultures of endothelial cell lines such as HUVEC and EA.hy926 are used extensively to investigate the beneficial effects of quercetin (derivatives) on endothelial (dys)function. For example, the protective effects of the flavonoids tricin, baicalin, baicalein and wogonin against LPS-induced endothelium activation and inflammation in HUVEC cells was verified by Shalini et al. (2016) and W. Lee, Ku and

Bae (2015). The expression of adhesion molecules (ICAM-1, VCAM-1 and E-selectin) and inflammatory cytokines (MCP-1, TNF-α and IFN-γ) was upregulated by 1 µg/mL LPS and dose-dependently downregulated again by 1.5-30 µM tricin (Shalini et al., 2016). Baicalin, baicalein and wogonin (10 µM) showed to significantly ($p < 0.05$) inhibit 100 ng/mL LPS-induced upregulation of VCAM-1, ICAM-1, E-selectin, TNF-α and IL-6 (W. Lee et al., 2015). The effect of genistein and luteolin on TNF-α-stressed HUVEC and EA.hy926 cells, respectively, was assessed by Jia et al. (2013) and Jia et al. (2015). Genistein (0.1-10 µM) significantly ($p < 0.05$) reduced ICAM-1, VCAM-1, E-selectin, MCP-1 and IL-8 levels in HUVEC cells that were increased by 2 ng/mL TNF-α (Jia et al., 2013). Moreover, TNF-α-upregulated MCP-1, ICAM-1 and VCAM-1 expression was significantly ($p < 0.05$) downregulated by luteolin (0.1-2 µM) in EA.hy926 cells. In addition, luteolin (1-2 µM) was observed to counteract TNF-α-elevated NF-κB activation (Jia et al., 2015). At a concentration of 50 µM, quercetin was capable of reducing the protein levels of adhesion molecules ICAM-1, VCAM-1 and E-selectin – measured by densitometric analysis of Western blot – in HUVECs that were pre-treated with a cytokine mixture containing TNF-α, IFN-γ and IL-1β. Furthermore, at a concentration of 1-50 µM, quercetin dose-dependently reduced iNOS expression (Crespo et al., 2008).

1.4.2.1.3 Monocytes and macrophages

The effect of quercetin was also tested on monocyte cell line cultures. Both Y. Lu and Jia (2016) and L. Sun et al. (2015) concluded based on liquid scintillation counting assays that cholesterol efflux is increased by quercetin in THP-1 cells, thereby inhibiting foam cell formation. Gene expression of IL-1β, IL-6, IL-8 and NF-κB in THP-1 monocytes was observed to be upregulated after exposure to 700 ng/mL LPS and downregulated again after administration of 50 µM quercetin. It should be noted that gene expression of TNF-α was upregulated after LPS administration, but not downregulated by quercetin (Chanput, Mes, Vreeburg, Savelkoul, & Wichers, 2010).

As mentioned before, NO produced by endothelial cells is crucial for vasodilation. However, iNOS in macrophages can produce high amounts of NO which can cause oxidative damage. When NO reacts with free radicals, it can form the highly reactive peroxynitrite which in turn oxidizes LDL. Quercetin is able to scavenge free radicals and NO, thereby reducing cell damage (Nijveldt et al., 2001). The inhibition of LPS-induced iNOS gene expression in RAW264.7 macrophages by quercetin was demonstrated in a study of Y. C. Chen et al. (2001). A significant reduction ($p < 0.01$) in iNOS gene expression was observed in macrophages treated with LPS and 20 or 40 µM quercetin, compared to cells that were only treated with LPS (Y. C. Chen et al., 2001). A similar study of H. N. Lee et al. (2018) concluded that 12.5-25 µM quercetin reduced LPS-induced NO production in RAW264.7 macrophages significantly ($p < 0.05$). H. P. Kim et al. (2004) reviewed several studies that investigated the effect of flavonoids on the expression of pro-inflammatory genes. It was resumed that several flavonoids are capable of downregulating the TNF-α/LPS/IL-1β-induced expression of inflammatory genes such as TNF-α, IL-6, IL-8 and IL-1β in endothelial cells (HUVEC), monocytes (human peripheral blood mononuclear cells) or macrophages (RAW.264.7) (H. P. Kim et al., 2004). These findings were in line with the conclusions of the studies described above.

1.4.2.2 Co-culture models used to investigate flavonoid bioavailability and bioactivity towards endothelial dysfunction and atherosclerosis

1.4.2.2.1 Intestinal-endothelial co-culture models

Peripheral cells can be co-cultured with intestinal cells that will absorb and metabolize quercetin before reaching the former cells. In addition, these types of co-culture models allow crosstalk between intestinal cells and the peripheral endothelial cells which simulate the *in vivo* situation better.

Jiang et al. (2019) described a non-contact co-culture model where Caco-2 cells were cultured on the apical side of a Transwell®, whereas EA.hy926 cells were cultured on the basolateral compartment of the Transwell®. Pre-incubation with red-osier dogwood extracts in the apical compartment, followed by treatment of the endothelial cell line in the basolateral compartment with TNF-α, seemed to result in a reduction of IL-8, TNF-α, ICAM-1 and VCAM-1 expression. The same co-culture model was used by Toaldo et al. (2016) and Kamiloglu et al. (2017) to demonstrate the beneficial effect of the polyphenol resveratrol on TNF-α-induced endothelial dysfunction and the anti-inflammatory potential of polyphenols in a black carrot, respectively. In the former study, TNF-α (10 ng/mL) increased the production of IL-8 and ICAM-1, whereas this effect was opposed by resveratrol (5-20 µM) (Toaldo et al., 2016). Similar results were observed in the second study where polyphenols in a black carrot reduced 1 ng/mL TNF-α-induced IL-8, MCP-1 and ICAM-1 secretion (Kamiloglu et al., 2017).

The anti-inflammatory effect of anthocyanins was also implied in a non-contact co-culture model of Caco-2 cells – that are seeded on the apical compartment of a Transwell® plate – and HUVEC cells – that are seeded on the basolateral compartment. Anthocyanins decreased E-selectin, ICAM-1 and VCAM-1 expression in 1 ng/mL TNF-α-stimulated endothelial cells (Kuntz et al., 2015). Ferrari et al. (2017) described the same co-culture model with Caco-2 cells and HUVECs. The latter was established to evaluate the effect of the flavonoid cyadinin-3-o-glucoside on the *in vitro* inflammatory crosstalk between intestinal and endothelial cells. Briefly, Caco-2 cells were pre-treated with 20 µM cyanidin-3-o-glucoside before being stressed with 50 ng/mL TNF-α, after which they were assembled with HUVEC cells. Afterwards, a HUVEC-leukocyte co-culture was performed to assess leukocyte adhesion as a result of endothelial activation. It was observed that the mRNA of TNF-α and IL-8 was upregulated in Caco-2 cells by TNF-α and the upregulation was significantly lower after exposure to cyanidin-3-o-glucoside. HUVECs that had been in co-culture with TNF-α stressed Caco-2 cells showed an increase in E-selectin and VCAM-1 expression, compared to untreated cells. Leukocyte adhesion was enhanced as well. These effects were significantly opposed by pre-treatment of the Caco-2 cells with cyadinin-3-o-glucoside (Ferrari et al., 2017). Finally, a non-contact co-culture model with Caco-2 and mouse intestinal microvascular endothelial cells (MIMVEC) was set-up by Bian et al. (2020) in order to investigate the effect of kaempferol on inflammation induced by basolateral LPS. Kaempferol (80 µM) was observed to reduce 1 µg/mL LPS-upregulated IL-8 secretion in the apical compartment. Moreover, LPS-increased phosphorylation levels of the NF-κβ p65 subunit in Caco-2 cells were downregulated by kaempferol. Barrier integrity of the Caco-2 monolayer was negatively affected by LPS and restored by kaempferol (Bian et al., 2020).

1.4.2.2.2 Intestinal-monocyte/macrophage co-culture models

To evaluate the effect of absorbed and metabolized polyphenols on innate immune cells, a co-culture model of intestinal cells and monocytes/macrophages can be applied. Zhang et al. (2017) reported that anthocyanins from purple potato extract, transported by intestinal epithelial cells, counteracted the 15 ng/mL LPS-induced production of TNF-α and IL-8 by THP-1 macrophages. These results were accomplished using a Transwell® system with the apical compartment containing Caco-2 cells and the basolateral compartment containing activated THP-1 macrophages. The downregulation of the expression of inflammatory mediators in intestinal cells and macrophages that were stimulated with 5 ng/mL LPS by blackcurrant extracts containing polyphenols was observed by Olejnik et al. (2016) using a co-culture model of Caco-2 cells and RAW264.7 macrophages.

1.4.2.2.3 Endothelial-monocyte/macrophage co-culture models

Monocyte adhesion and transmigration assays were performed by Fuior et al. (2019) in order to examine the altered monocyte recruitment in response to 50 µM naringenin or hesperetin administration to TNF-α-treated endothelial cells. Therefore, fluorescently labelled THP-1 cells were added to 24 wells containing the treated EA.hy926 cells. After incubation, the adherent/migrated THP-1 cells were collected and fluorescence intensity was measured using a spectrofluorometer. The migration and adhesion of monocytes appeared to be significantly lower ($p < 0.001$) after flavonoid administration. A similar experiment was exerted by K. J. Kim et al. (2019) to investigate the effect of a plant extract containing flavonoids on THP-1 monocyte adhesion to TNF-α-treated EA.hy926 cells. A significantly lower ($p < 0.05$) adhesion was observed in the cells treated with plant extract. A monocyte adhesion assay using U937 monocytes was performed with human aortic endothelial cells (HAEC) to reveal the effect of quercetin. It was observed that pre-exposure of HUVECs to 1 or 10 µM quercetin reduced the adhesion of monocytes to 10 ng/mL IL-1β-treated HUVECs. Only a concentration of 10 µM quercetin showed a significant ($p < 0.01$) reduction (Koga & Meydani, 2001).

1.4.3 Critical remarks concerning current *in vitro* models used to investigate flavonoid bioavailability and bioactivity towards endothelial dysfunction and atherosclerosis

Overall, it can be concluded for clinical trials that the beneficial effects of flavonoids towards endothelial dysfunction and atherosclerosis are often detected in diseased subjects and at-risk populations, such as smokers, indicating the importance of the impact of health status on flavonoid responses. In line with this, *in vitro* studies concerning flavonoid bioactivity are performed under stressed conditions.

Regarding the applied pro-inflammatory stimuli, there is often a disparity between *in vivo* physiological concentrations and *in vitro* applied concentrations (Turner et al., 2010). TNF-α levels in the serum are mainly reported to be below 100 pg/mL (Page, Bester, & Pretorius, 2018). However, in case of inflammation, the amount of TNF-α can reach levels in the range of several hundred pg/mL (Damas et al., 1992). *In vitro* applied doses often exceed these ranges. In many *in vitro* studies, cells were stressed with 10 ng/mL TNF-α to induce high-grade inflammation and 1 ng/mL TNF-α to induce low-grade inflammation (Kamiloglu et al., 2017; Kuntz et al., 2015; Toaldo et al., 2016). Since atherosclerosis is

associated with low-grade inflammation, studies using low concentrations (≤ 1 ng/mL) TNF-α are more relevant. As regards LPS, physiological relevant concentrations range between 0 and 1 ng/mL and can be elevated to 10 ng/mL in diseased conditions (S. Guo, Al-Sadi, Said, & Ma, 2013). However, the LPS concentrations applied in the *in vitro* studies described above, often exceed this range. Furthermore, *in vivo*, the stress is chronically present, while cells are only stressed for a few hours (1-24h) in *in vitro* experiments.

Notably many of the *in vitro* studies are not taking the low flavonoid bioavailability and extensive flavonoid metabolism into account. Often flavonoid aglycones in concentrations far higher than physiological achievable are used against the guidelines described by Balentine et al. (2015). To perform *in vitro* flavonoid research under more physiological conditions, development of co-culture models where the flavonoid aglycone is first transported and transformed by intestinal cells prior to reaching the peripheral cells, is stimulated. Moreover, studies using plant extracts containing only a fraction of flavonoids are not useful for mechanistic research. After all, due to the presence of other (undefined) components in the extract, the particular effect of the flavonoids cannot be elucidated. Therefore, in order to unravel the mechanistic pathways behind the beneficial effect of flavonoids, the use of pure flavonoids in *in vitro* studies is recommended.

As demonstrated in this section, several studies have developed intestinal-endothelial, intestinal-immune or endothelial-immune co-culture models, however, a triple culture of endothelial, intestinal and immune cells – that would serve as a more physiological relevant model due to the ability of crosstalk between all the essential cell types involved in flavonoid bioavailability and the pathogenesis of atherosclerosis – is not described in literature so far.

2. PROBLEM STATEMENT AND OBJECTIVES

In this thesis, the beneficial effect of quercetin on endothelial dysfunction is explored. However, before investigating bioactivity, bioavailability must be taken into account. Therefore, intracellular quercetin accumulation in a stressed intestinal (Caco-2) and endothelial (EA.hy926) cell line is analysed, as depicted in Figure 7, which gives an overview of the research strategies and methods in this thesis. Intracellular quercetin accumulation is already investigated upon inflammatory stress, induced by LPS and TNF-α, in a previous study by Vissenaekens et al. (2019), therefore, the effect of stress hormones adrenaline and cortisol was looked into in this thesis. As described in the literature study of this thesis (section 1.1.5), these stressors are, just as LPS and TNF-α, linked with the pathogenesis of atherosclerosis.

The aim of this thesis was to develop novel triple co-culture models for endothelial dysfunction and atherosclerosis including intestinal, endothelial and immune cells. As discussed in section 1.4.2.2 of the literature study, several studies have developed co-culture models combining two of these cell types. However, there are no reports on the use of a triple co-culture with all three cell types in the context of atherosclerosis so far. Interestingly, a triple co-culture model for atherosclerosis containing endothelial cells, monocytes and smooth muscle cells was developed by Noonan et al. (2019). The crosstalk between endothelial cells and monocytes is a crucial event in the onset of atherosclerosis. However, as raised by Kroon et al. (2004), in many *in vitro* studies, quercetin aglycone or polyphenol-rich extracts are applied to cells in non-physiological concentrations. First of all, the absorption and metabolism of these compounds, rendering quercetin metabolites with a potentially dissimilar bioactivity, is often not taken into account. Second, peak quercetin levels in the blood rage between 0-10 µM, which is often exceeded in *in vitro* studies (Kroon et al., 2004). In order to tackle these shortcomings, an intestinal barrier is included in the model set-up of this thesis. In the triple co-cultures, quercetin is applied to the intestinal barrier and endothelial dysfunction is provoked by LPS. This stressor was chosen due to the acquired knowledge concerning LPS that is extensively described in literature and the numerous results of experiments using LPS on mono- and co-cultures.

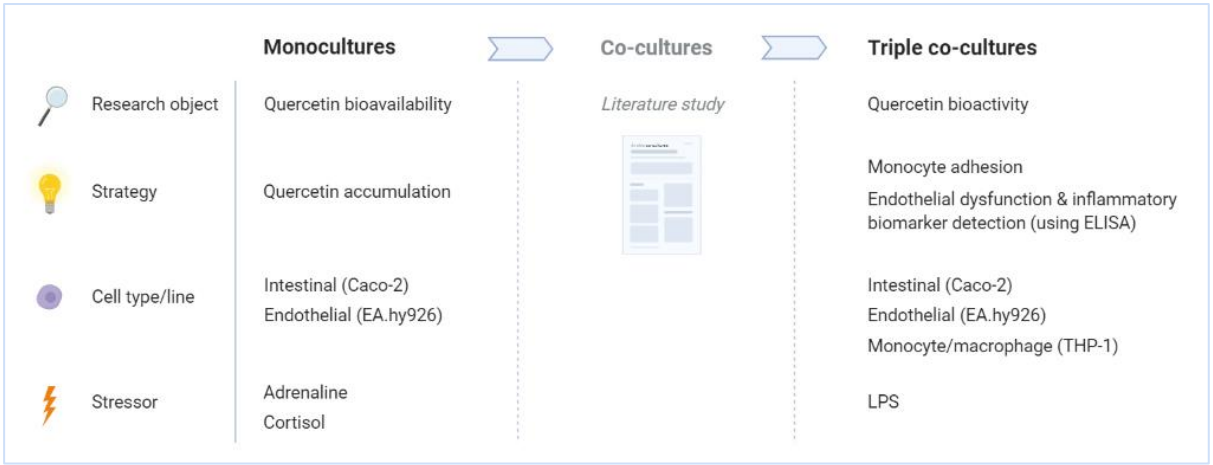


Figure 7. Overview of objectives and methods in this thesis

3. MATERIALS AND METHODS

3.1 Reagents and chemicals

The human intestinal epithelial cell line Caco-2, the human endothelial cell line EA.hy926 and the human monocytic cell line THP-1 were obtained from the American Type Culture Collection (ATCC). The cell culture media (Dulbecco's Modified Eagle's Medium (DMEM) 1 g/L glucose, DMEM 4.5 g/L glucose and RPMI 1640), non-essential amino acids (NEAA), penicillin/streptomycin (P/S), trypsin-EDTA and sterile phosphate buffered saline (PBS) without calcium and magnesium were purchased from Gibco (Merelbeke, Belgium). Non-sterile PBS tablets, 0.25% (v/v) trypsin-EDTA solution, paraformaldehyde, ethanolamine, Triton-X100, 2-aminoethyl-diphenylborinate, dimethylsulfoxide (DMSO), resazurin, trichloroacetate (TCA), trishydroxymethylaminomethane (Tris), sulforhodamine B (SRB), Fluoroshield Mounting Medium, quercetin, adrenaline, cortisol, ascorbic acid and LPS were bought from Sigma-Aldrich (Diegem, Belgium). Trypan blue and ethanolamine were obtained from VWR (Leuven, Belgium) and Janssens Chimica (Beerse, Belgium), respectively. Glacial acetic acid was purchased from Fisher Scientific (Merelbeke, Belgium) and CellTrace Calcein Violet AM was bought from Invitrogen (Merelbeke, Belgium).

3.2 Cell culture

The Caco-2 and EA.hy926 cells were cultivated in T-75 flasks using growth medium DMEM containing GlutaMax, pyruvate, 4.5 g/L glucose, 1% NEAA, 1% P/S and 10% fetal bovine serum (FBS) and were maintained in an incubator (37°C, 10% CO_2, humidified atmosphere). The THP-1 cells were cultivated in RPMI 1640 supplemented with 1% P/S and 10% FBS in a humidified incubator (37°C, 10% CO_2). The growth medium was replaced every other day and the cells were sub-cultivated using 0.25 % (v/v) tryspin-EDTA solution when confluency reached 80%.

3.3 Experimental set-up

3.3.1 Monocultures

An overview of the experimental workflow of monoculture studies that were used to investigate cytotoxicity and the intracellular quercetin accumulation, is given in Figure 8.

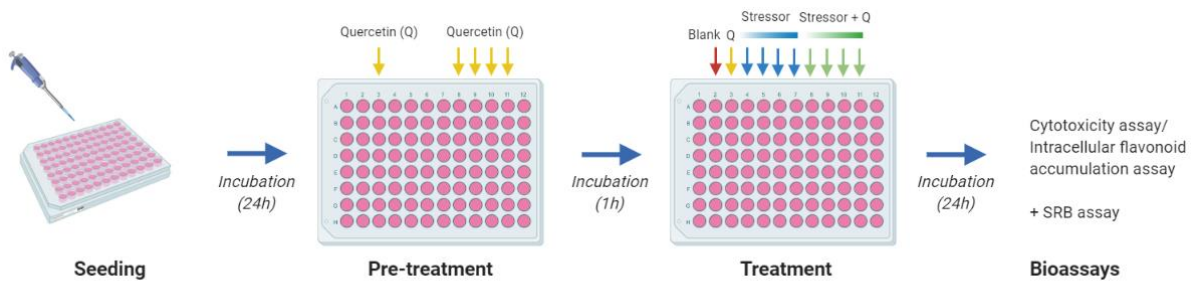


Figure 8. Experimental workflow of monoculture models

3.3.1.1 Cytotoxicity assay

Caco-2 cells and EA.hy926 cells were both seeded using growth cell culture medium in transparent 96-well plates at a density of 60 000 cells/cm^2. Cells were counted using a Bürker counting chamber and trypan blue. Twenty-four hours post-seeding, the EA.hy926 and Caco-2 cells were treated with the stressor of interest, with or without co-administration of quercetin, using FBS-free cell culture medium. Treatment of quercetin was preceded by a pre-treatment with quercetin for 1 hour. The concentrations of quercetin, adrenaline and cortisol that were applied for each cell line are listed in Table 2. After incubation (37°C, 10% CO_2, humidified atmosphere) for 24 hours, the medium was aspirated from the cells and resazurin (10 µg/mL in FBS-free cell culture medium) was applied to each well. Resazurin can be reduced to fluorescent resorufin by metabolic active cells, therefore, the latter can be measured as an indicator for cell viability (Riss et al., 2004). After incubation for 2 hours at 37°C, the supernatants was transferred to a black 96-well plate and fluorescence (560 nm excitation, 590 nm emission) was measured using Spectramax M2 Multimode Plate Reader. All measurements were corrected for background medium fluorescence.

Table 2. Concentrations of quercetin and stressors applied for each cell line

Cell line	Quercetin	Adrenaline	Cortisol
Caco-2	10 µM	1 µM, 10 µM, 20 µM	10 nM, 100 nM, 500 nM, 1 µM
EA.hy926	3 µM	1 nM, 10 nM, 100 nM, 1 µM	10 nM, 100 nM, 500 nM, 1 µM

3.3.1.2 Detection of intracellular flavonoid accumulation

Cells were seeded in black 96-well plates and treated in the same way as described in section 3.3.1.1. After incubation (37°C, 10% CO_2, humidified atmosphere) of the plates for 24 hours, cells were fixed with paraformaldehyde (4 % in PBS) for 12 hours at 4°C. Afterwards, residues of paraformaldehyde were removed using 10 mM ethanolamine in PBS and cells were washed with PBS. After permeabilization of the cells using 0.5% Triton-X100 in PBS, cells were washed again with PBS and stained with 2-aminoethyl-diphenylborinate (0.2% w/v in 3% DMSO in distilled water). One replicate of each condition was loaded with 3% DMSO in H_2O to correct for background fluorescence. After 2 hours, cells were washed with PBS. Then 200 µL of PBS was added to each well and fluorescence (485 nm excitation, 520 nm emission) was measured using Spectramax M2 Multimode Plate Reader. A risk analysis was performed on the use of chemical agents required for the intracellular flavonoid accumulation (Supplementary data A).

3.3.1.3 Protein measurement

The results of the cytotoxicity assay (section 3.3.1.1) and the flavonoid accumulation assay (section 3.3.1.2) must be corrected for the amount of cells in each well. For this, the cellular protein content serves as an estimation for cell count and is measured by a SRB colorimetric assay. The SRB dye binds electrostatically and pH-dependant on basic amino acids of cellular proteins (Vichai & Kirtikara, 2006).

After the cytotoxicity assay (section 3.3.1.1), cells were fixed using TCA (50% in milliQ-water) for 1 hour at 4°C. Thereafter, the cells were rinsed with water, dried and subsequently stained with SRB solution (0.4 % (w/v) in 1% glacial acetic acid). After 30 minutes, the cells were rinsed with glacial acetic acid (1% in milliQ-water) to remove unbound SRB. 200 µL of 10 mM Tris buffer was loaded in each well and absorbance was measured at 490 nm using Spectramax M2 Multimode Plate Reader.

Cells that were already fixed with paraformaldehyde to determine flavonoid accumulation (section 3.3.1.2), could directly be stained with SRB stain for 30 minutes. Thereafter, the cells were rinsed with glacial acetic acid (1% in milliQ-water) and 200 µL of 10 mM Tris buffer was loaded in each well. Finally, the absorbance was measured at 490 nm using Spectramax M2 Multimode Plate Reader.

3.3.2 Triple co-cultures

In the present thesis, three triple co-culture models were developed, as illustrated in Figure 9.

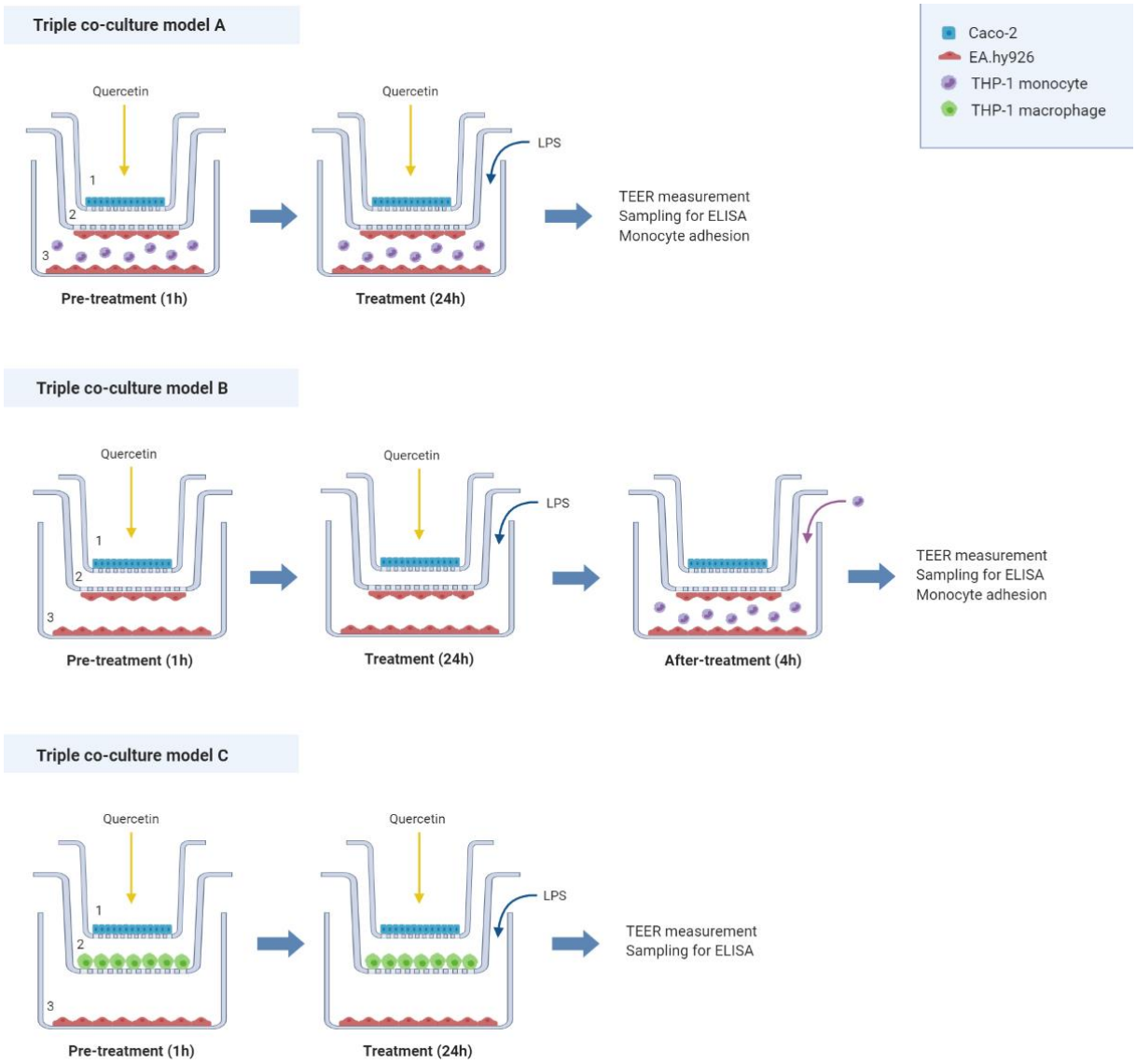


Figure 9. Experimental set-up of three triple co-culture models. Compartments are numbered: apical (1), interstitial (2) and basal (3) compartment.

For model A, Caco-2 cells were seeded on the apical side of the Transwell® membrane of a transparent 12-well Transwell® plate (compartment 1) at a density of 60 000 cells/cm^2 in growth cell culture medium and maintained for 3 weeks to obtain differentiated Caco-2 cells.
EA.hy926 cells were seeded on the basal side of the Transwell® membrane and in the basal compartment of two transparent 6-well Transwell® plates (compartment 2) at a density of 60 000 cells/cm^2 in growth medium. After one week of maintenance, the growth medium was replaced by FBS-free cell culture medium without NEAA supplementation (quiescent medium) and maintained for another 24 hours in the incubator, in order to obtain quiescent EA.hy926 cells.
THP-1 cells were stained with CellTrace Calcein Violet AM (1µM in 1 g/L glucose DMEM without NEAA and FBS) for 30 minutes. Subsequently, they were washed with PBS and resuspended in 1 g/L glucose DMEM without NEAA and FBS to a final density of 200 000 cells/mL. Thereafter, the cell suspension was administered to compartment 3 of model A.

As summarized by Table 3, the different compartments of model A receive different growth medium with a decreasing sugar concentration and increasing serum concentration from the intestinal to the endothelial compartment. Table 4 shows that the intestinal compartment was pre-treated (1h) and subsequently exposed (24h) with quercetin, whereas the endothelial compartment was exposed (24h) to the endotoxin LPS.

The intestinal and endothelial compartment of model B are similar to model A, however the THP-1 cells are only administered to compartment 3 after exposure (24h) with quercetin and/or LPS. Another difference is the presence of FBS in the medium of compartment 3 during (pre-)treatment. There is no FBS in the medium, containing THP-1 cells, that is administered to the basal compartment after treatment, in order to prevent interference of the serum in the ELISA assay. An overview of the treatment set-up of model B is displayed in Table 5 and 6.

Due to measures taken because of Covid-19, experiments using model C could not be executed in the lab. Therefore, this model will be explained in more detail in the section Future perspectives.

3.3.2.1 Barrier integrity

Barrier integrity of Caco-2 and EA.hy926 monolayers, cultured on the semipermeable Transwell® membranes, were determined by transepithelial/transendothelial electrical resistance (TEER) measurements before and after treatment using the REMS AutoSampler. TEER (Ω*cm^2) is the product of the measured tissue resistance (Ω) multiplied with the area of the semipermeable membrane (cm^2) (Srinivasan et al., 2015). TEER values of EA.hy926 and Caco-2 monolayers were measured in quiescent medium and growth cell culture medium, respectively. The result was expressed as percentages of the ratio of TEER values after/before treatment, in order to evaluate the effect of the treatment on the barrier integrity of the monolayers.

3.3.2.2 Monocyte adhesion assay

As described before, THP-1 cells were stained with CellTrace Calcein Violet AM before introduction to compartment 3 of the triple co-culture. This fluorescent Calcein Violet dye is unable to cross a cell membrane unless it is complexed with acetoxymethyl (AM) that neutralizes the molecule. Non-fluorescent Calcein Violet AM can be taken up in cells and is captured in viable cells by the cleavage of AM ester groups by intracellular esterases, resulting in a fluorescent molecule (Miles, Lynch, & Sikes, 2015).

After the treatment steps, the medium in the basal compartments was removed and the EA.hy926 cells with adherent THP-1 cells were washed and mounted on a microscopic glass slide containing Fluoroshield Mounting Medium. For each well, 9 images in a grid from the basal monolayer and 6 images from the filter membrane were taken with a fluorescent microscope (405 nm excitation, 450/50nm emission). Fluorescent THP-1 cells on each image were counted using ImageJ.

3.3.2.3 ELISA assay

After exposure to quercetin and/or LPS, samples from all compartments were taken and centrifuged. The supernatants was stored at -80°C.

Due to measures taken because of Covid-19, the ELISA assay could not be performed in the lab. This assay will be discussed in the section Future perspectives.

3.4 Statistical analysis

Statistical analysis was performed on all data. In order to detect significant differences between several groups and a control group, a one-way ANOVA was carried out in R software. Significance between two groups (e.g. a condition with and without quercetin administration) was evaluated using a heteroscedastic two-tailed t-test in Microsoft Excel.

Table 3. An overview of the growth media applied to the different compartments of model A during (pre-)treatment

	Pre-treatment (1h)		**Treatment (24h)**	
Compartment	4.5 g/L glucose DMEM supplemented with 1% (v/v) NEAA	1 g/L glucose DMEM	4.5 g/L glucose DMEM supplemented with 1% (v/v) NEAA	1 g/L glucose DMEM
Compartment 1	✓	×	✓	×
Compartment 2	×	✓	×	✓
Compartment 3	×	✓	×	✓

Table 4. An overview of quercetin and LPS administration to compartment 1 and 3 of model A during (pre-)treatment

	Pre-treatment (1h)		**Treatment (24h)**	
Condition	10 µM Quercetin (compartment 1)	10 ng/mL LPS (compartment 3)	10 µM Quercetin (compartment 1)	10 ng/mL LPS (compartment 3)
Untreated	×	×	×	×
LPS	×	×	×	✓
Quercetin	✓	×	✓	×
LPS + Quercetin	✓	×	✓	✓

Table 5. An overview of the growth media applied to the different compartments of model B during (pre-/after-)treatment

	Pre-treatment (1h)			**Treatment (24h)**			**After-treatment (4h)**	
Compartment	4.5 g/L glucose DMEM supplemented with 1% (v/v) NEAA	1 g/L glucose DMEM	1 g/L glucose DMEM supplemented with 10% (v/v) FBS	4.5 g/L glucose DMEM supplemented with 1% (v/v) NEAA	1 g/L glucose DMEM	1 g/L glucose DMEM supplemented with 10% (v/v) FBS	4.5 g/L glucose DMEM supplemented with 1% (v/v) NEAA	1 g/L glucose DMEM
Compartment 1	✓	×	×	✓	×	×	✓	×
Compartment 2	×	✓	×	×	✓	×	×	✓
Compartment 3	×	×	✓	×	×	✓	×	✓

Table 6. An overview of quercetin and LPS administration to compartment 1 and 3 of model B during (pre-/after-)treatment

	Pre-treatment (1h)		**Treatment (24h)**		**After-treatment (4h)**	
Condition	10 µM Quercetin (compartment 1)	10 ng/mL LPS (compartment 3)	10 µM Quercetin (compartment 1)	10 ng/mL LPS (compartment 3)	10 µM Quercetin (compartment 1)	10 ng/mL LPS (compartment 3)
Untreated	×	×	×	×	×	×
LPS	×	×	×	✓	×	×
Quercetin	✓	×	✓	×	×	×
LPS + Quercetin	✓	×	✓	✓	×	×

4. RESULTS

4.1 Monocultures

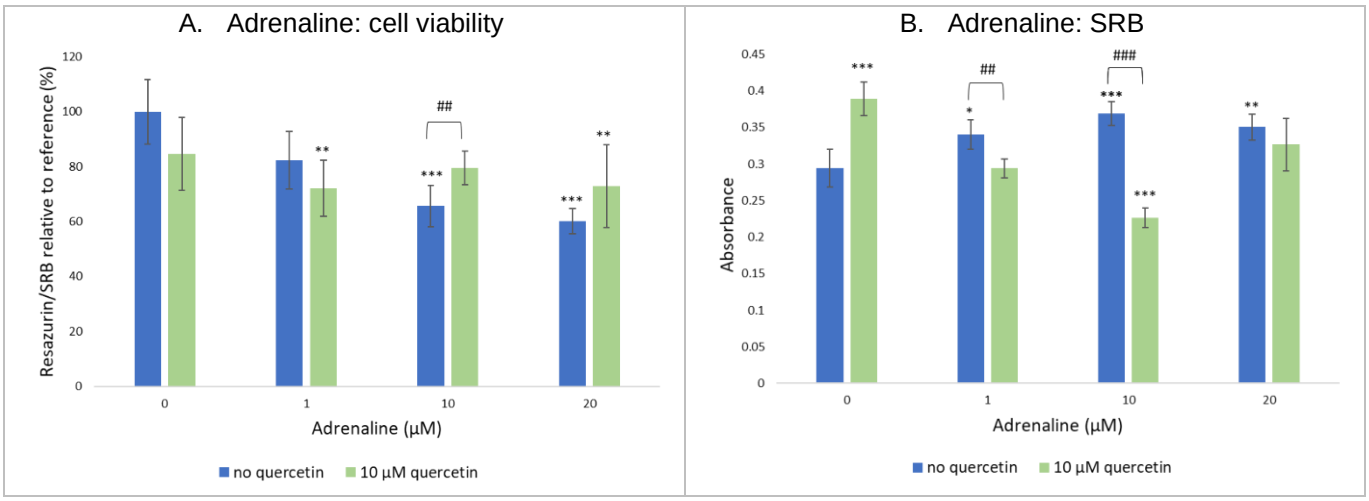
4.1.1 Determination of cytotoxicity of adrenaline and cortisol in Caco-2 and EA.hy926

Prior to the determination of flavonoid accumulation in adrenaline- and cortisol-stressed cells, it had to be assured that the proposed concentration ranges of stressors did not cause excessive cytotoxicity. Therefore, as a first step, cytotoxicity of adrenaline and cortisol, was assessed on both Caco-2 as EA.hy926 cells. Furthermore, the effect of quercetin on cytotoxicity was also evaluated on stressed and non-stressed cells. Data of the resazurin assay were divided by SRB data in order to correct for the amount of cells in each well. The data is represented in Figure 10 and 11.

The highest concentrations of adrenaline (10-20 µM) and all concentrations of cortisol cause a significant decrease in cell viability of Caco-2 cells, compared to the untreated cells. Interestingly, in combination with the stressors, quercetin increased cell viability of Caco-2 cells, compared to the stressed cells that were not treated with quercetin (Figure 10A, 10C). Also, the increase in protein content in Caco-2 cells of upon adrenaline stress is remarkable (Figure 10B).

For the EA.hy926 cells, there was no significant difference in resazurin/SRB values for both stressors, compared to the untreated cells (Figure 11A, 11C).

Although cell viability of Caco-2 cells dropped below 80% compared to the reference value (untreated) when stressed with adrenaline (> 10 µM) or cortisol (> 0.01 µM), it was decided to apply the same concentration ranges of both stressors to investigate their impact on intracellular flavonoid accumulation, since the co-administration with quercetin reduced the cytotoxicity. After all, it seemed interesting to explore a potential link between the positive effect of quercetin on cell viability and quercetin accumulation.



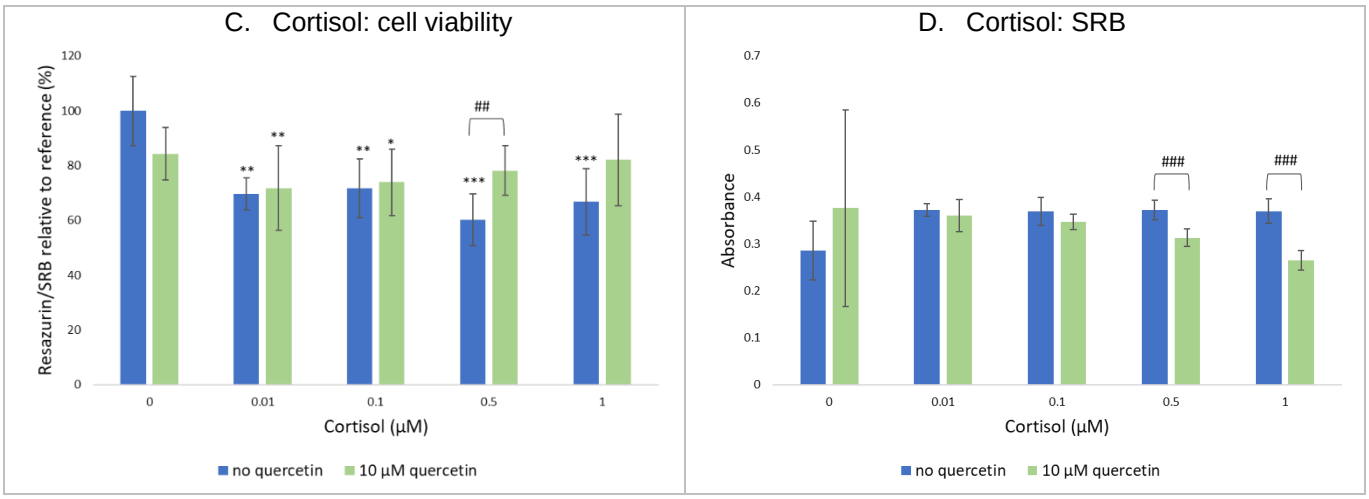


*Figure 10. Cell viability of Caco-2 cells treated with adrenaline, cortisol and/or quercetin (A,C). Resazurin data is corrected for protein content by SRB data (B,D). The bar plot displays the average of all replicates (n=6) and the standard deviation is represented by error bars. Significant differences with the reference (untreated cells) are indicated with * (p<0.05: *, p<0.01: **, p<0.001: ***). Significant differences between stressed cells treated with and without quercetin are indicated with # (p<0.05: #, p<0.01: ##, p<0.001: ###).*

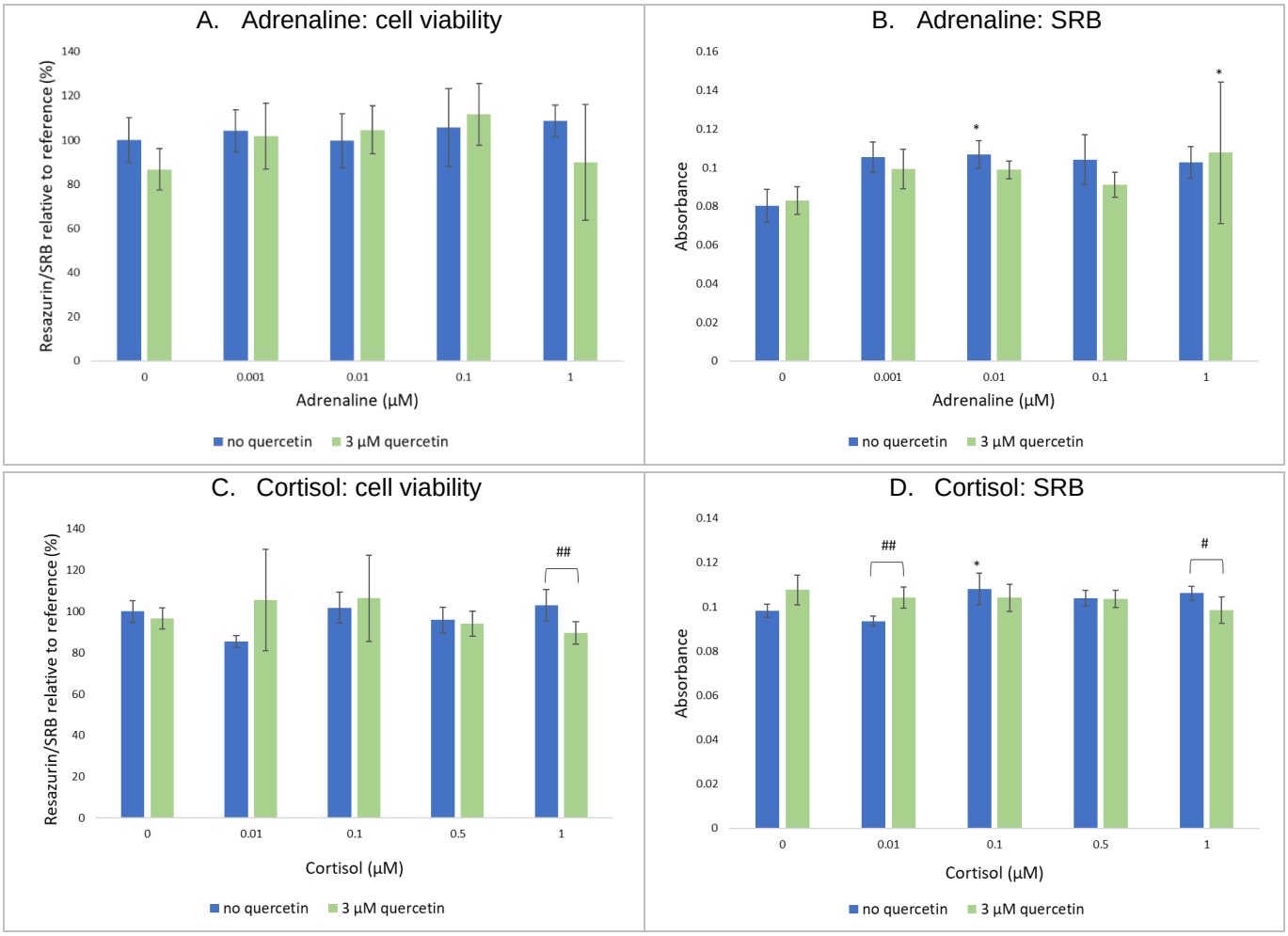


*Figure 11. Cell viability of EA.hy926 cells treated with adrenaline, cortisol and/or quercetin (A,C). Resazurin data is corrected for protein content by SRB data (B,D). The bar plot displays the average of all replicates (n = 6) and the standard deviation is represented by error bars. Significant differences with the reference (untreated cells) are indicated with * (p<0.05: *, p<0.01: **, p<0.001: ***). Significant differences between stressed cells treated with and without quercetin are indicated with # (p<0.05: #, p<0.01: ##, p<0.001: ###).*

4.1.2 Intracellular flavonoid accumulation in stressed Caco-2 and EA.hy926 cells

Intracellular flavonoid accumulation was examined in Caco-2 and EA.hy926 cells stressed with adrenaline and cortisol. When the intestinal and endothelial cells were exposed to quercetin under non-stressed conditions, a significant intracellular quercetin accumulation was observed (Figure 12A, 12C, 13A, 13C). Interestingly, intestinal cells stressed by 1 µM adrenaline, showed a significant higher intracellular quercetin accumulation compared to non-stressed intestinal cells (Figure 12A). A decreasing trend in quercetin accumulation upon increased stressor concentrations can be observed as well (Figure 12A, 12C). Upon cortisol stress (> 0.5 µM), the intracellular quercetin accumulation in the intestinal cells was significantly reduced compared to non-stressed intestinal cells (Figure 12C). For the endothelial cells, no unambiguous conclusions regarding the effect of stressors on quercetin accumulation could be drawn, since large variations are observed in those bar plots (Figure 13A, 13C).

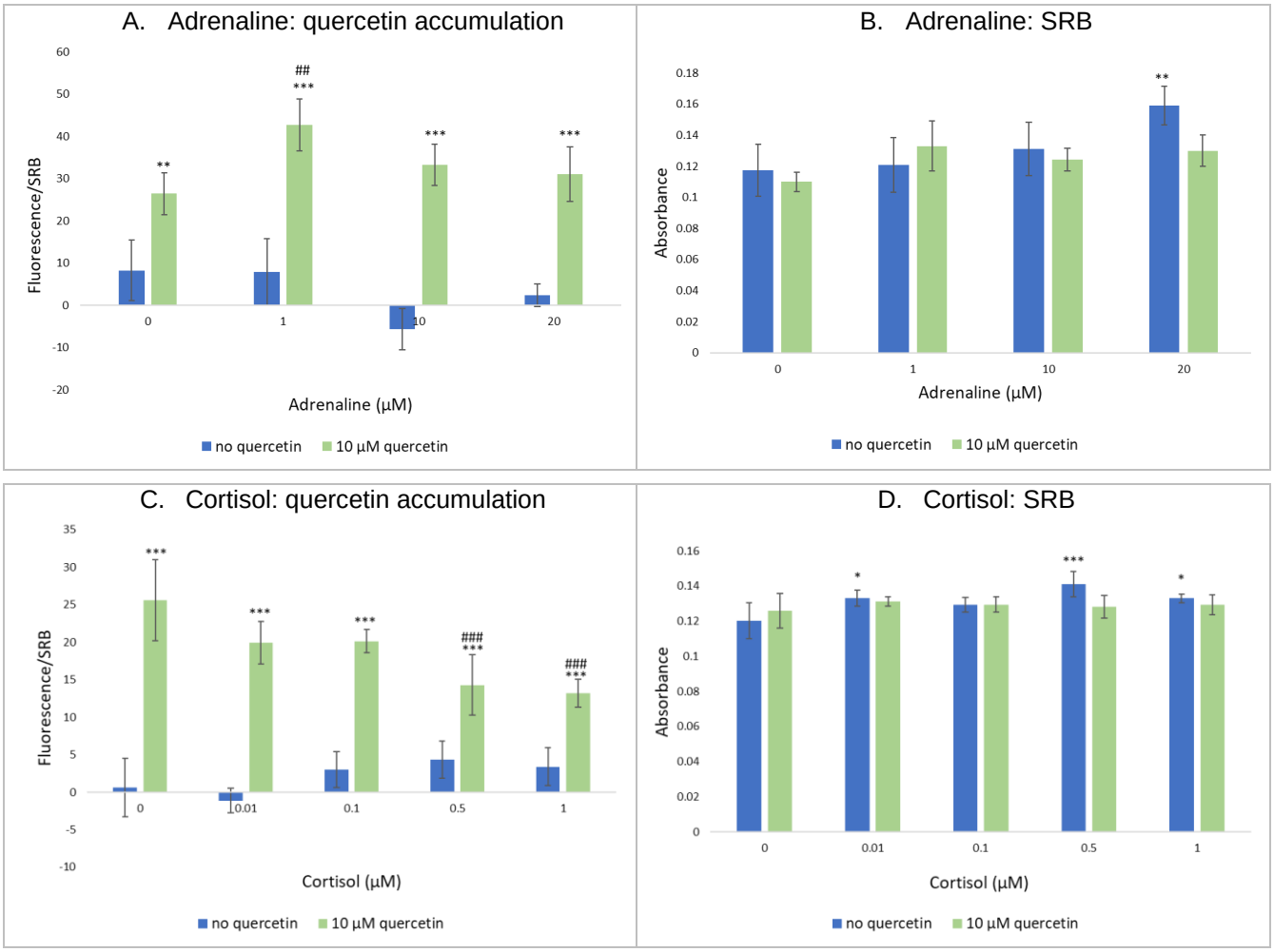


*Figure 12. Intracellular flavonoid accumulation in Caco-2 cells treated with adrenaline, cortisol and/or quercetin (A,C). Accumulation data is corrected for protein content by SRB data (B,D). The bar plot displays the average of all replicates (n = 5) and the standard deviation is represented by error bars. Significant differences with the reference (untreated cells) are indicated with * (p<0.05: *, p<0.01: **, p<0.001: ***). Significant differences between quercetin treated non-stressed cells and quercetin treated stressed cells are indicated with # (p<0.05: #, p<0.01: ##, p<0.001: ###).*

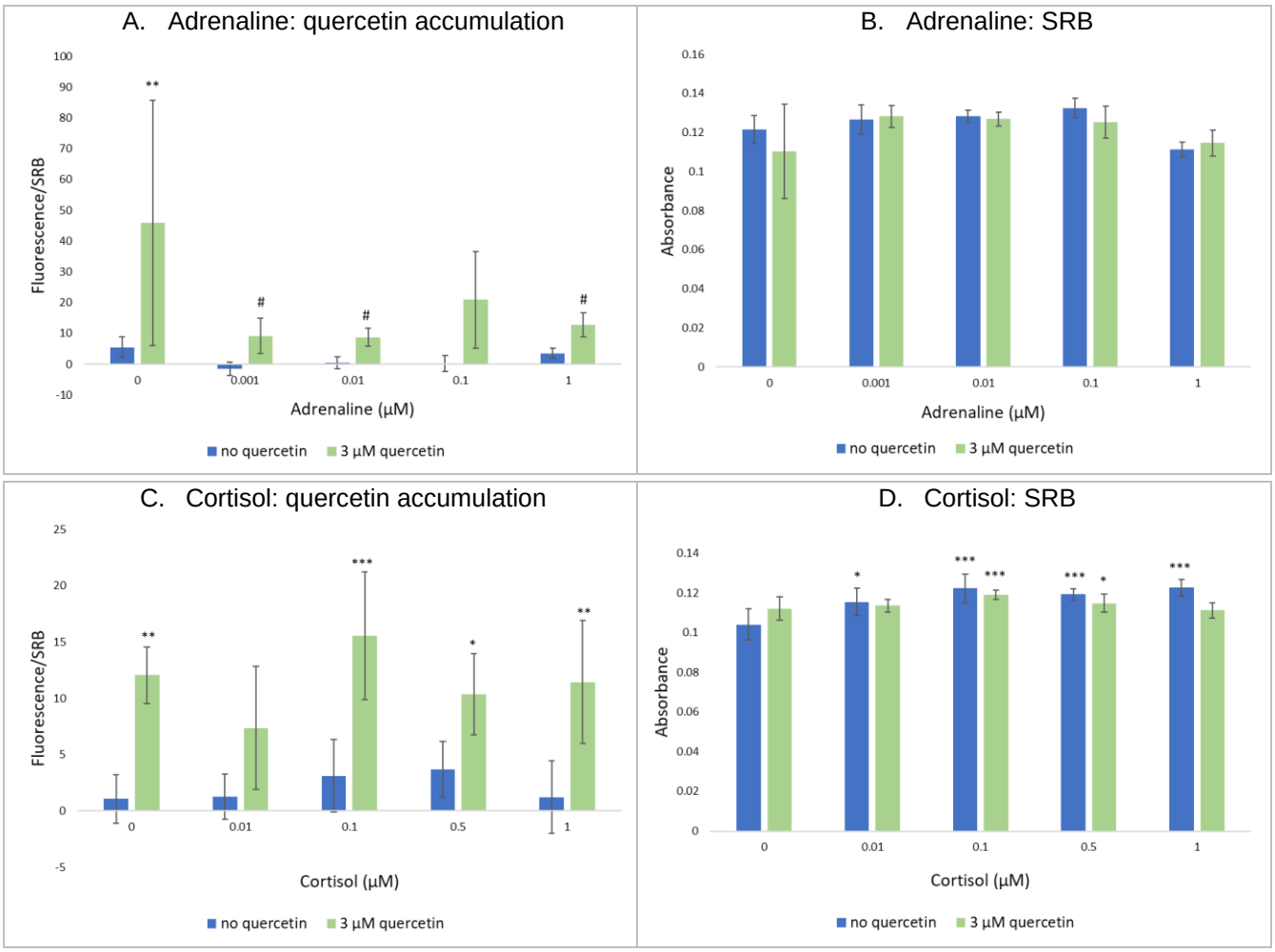


*Figure 13. Intracellular flavonoid accumulation in EA.hy926 cells treated with adrenaline, cortisol and/or quercetin (A,C). Accumulation data is corrected for protein content by SRB data (B,D). The bar plot displays the average of all replicates (n = 5) and the standard deviation is represented by error bars. Significant differences with the reference (untreated cells) are indicated with * (p<0.05: *, p<0.01: **, p<0.001: ***). Significant differences between quercetin treated non-stressed cells and quercetin treated stressed cells are indicated with # (p<0.05: #, p<0.01: ##, p<0.001: ###).*

4.1.3 Influence of ascorbic acid on quercetin stability

Bandaruk et al. (2014) suggested that quercetin is unstable in culture medium, due to its auto-oxidative properties. In the presence of ascorbic acid, i.e. an anti-oxidant, this effect seems to be counteracted. Therefore, in this thesis, the intracellular flavonoid accumulation assay was performed with 0.1 µM ascorbic acid administered to every wall, in addition to the original treatment, in order to consider major dissimilarities in the results.

As expected, quercetin treated intestinal cells accumulated significantly fluorescent-stained quercetin, compared to untreated cells (Figure 14A, 15A). No significant differences could be observed between the quercetin treated stressed and non-stressed cells, which is mainly due to the high variability observed for the non-stressed cells.

The signal of quercetin treated non-stressed Caco-2 cells was approximately the same when ascorbic acid was administered (Figure 14A) compared to when ascorbic acid was not supplemented (Figure 12A, 12C).
A similar result could be observed for EA.hy926 cells (Figure 13A, 13C, 15A). In Figure 13A, a large signal of quercetin treated non-stressed cells was seen, although it is accompanied by large error bars. Based on these observations, it cannot be concluded that potential quercetin degradation is counteracted by ascorbic acid.

In Figure 14B, the significant decrease in protein content in quercetin treated wells, compared to the other wells, is remarkable. An explanation can be found in an observation made by Bandaruk et al. (2014), namely that ascorbic acid, in combination with quercetin, may have cytotoxic effects, especially at high concentrations (500 µM ascorbic acid in combination with 10-50 µM quercetin). However, there were no significant differences between quercetin and non-quercetin groups in EA.hy926 (Figure 15B), which could suggest that these cells are not as sensitive to the potential cytotoxic effects of the ascorbic acid-quercetin mixture.

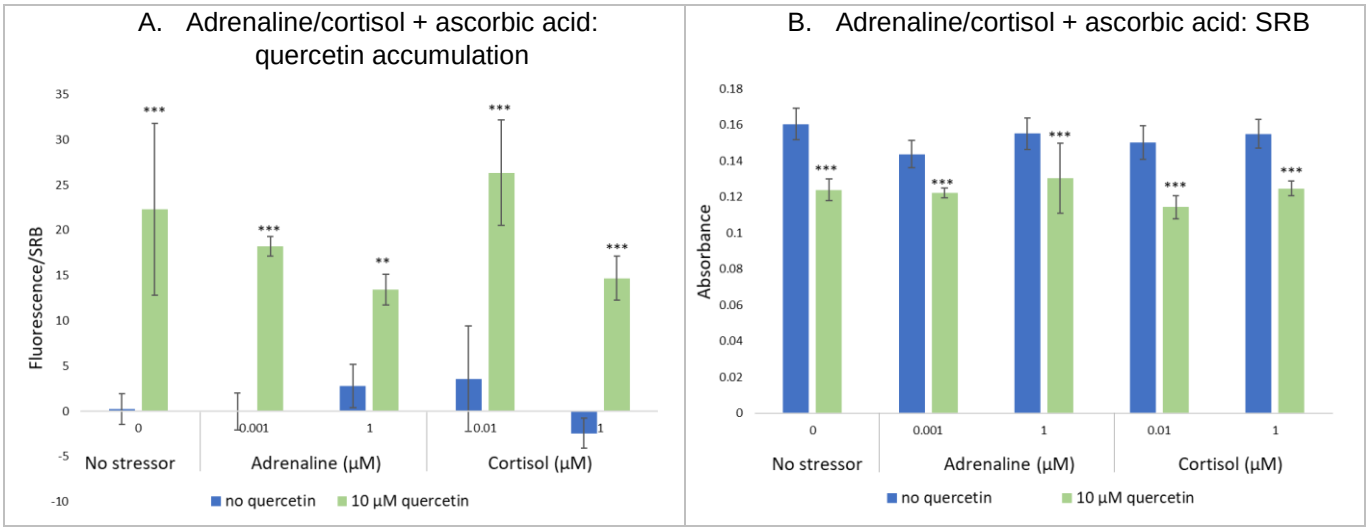


*Figure 14. Intracellular flavonoid accumulation in Caco-2 cells treated with adrenaline, cortisol and/or quercetin, in combination with 0.1 µM ascorbic acid (A). Accumulation data is corrected for protein content by SRB data (B). The bar plot displays the average of all replicates (n = 5) and the standard deviation is represented by error bars. Significant differences with the reference (untreated cells) are indicated with * (p<0.05: *, p<0.01: **, p<0.001: ***). Significant differences between quercetin treated non-stressed cells and quercetin treaded stressed cells indicated with # (p<0.05: #, p<0.01: ##, p<0.001: ###).*

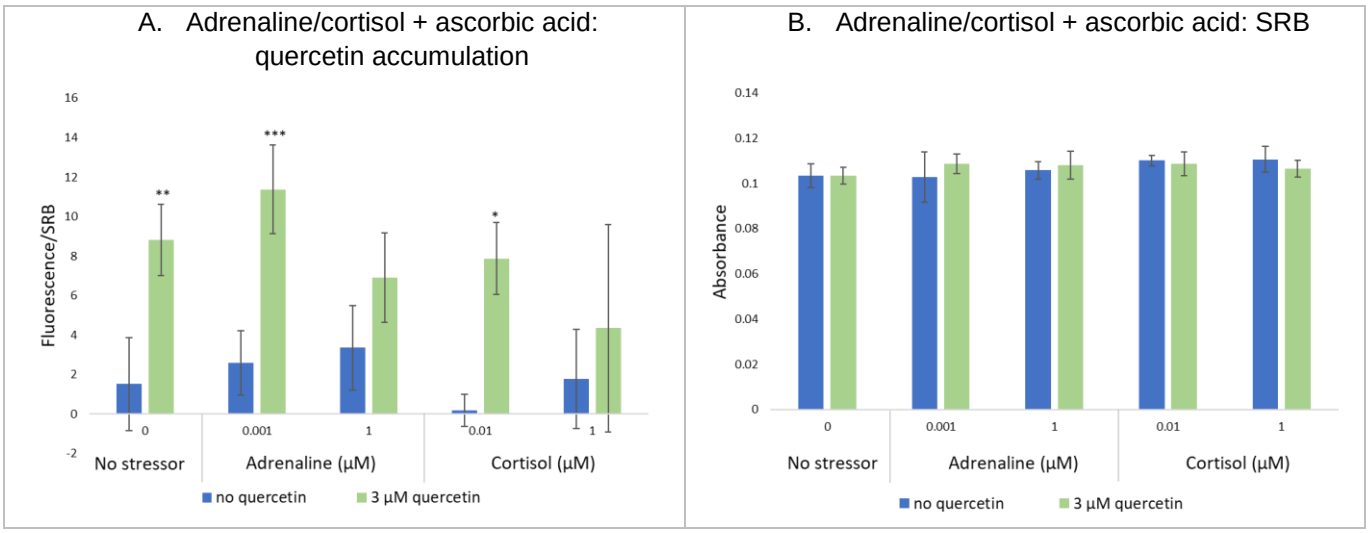


*Figure 15. Intracellular flavonoid accumulation in EA.hy926 cells treated with adrenaline, cortisol and/or quercetin, in combination with 0.1 µM ascorbic acid (A). Accumulation data is corrected for protein content by SRB data (B). The bar plot displays the average of all replicates (n = 5) and the standard deviation is represented by error bars. Significant differences with the reference (untreated cells) are indicated with * (p<0.05: *, p<0.01: **, p<0.001: ***). Significant differences between quercetin treated non-stressed cells and quercetin treaded stressed cells indicated with # (p<0.05: #, p<0.01: ##, p<0.001: ###).*

4.2 Triple co-cultures

4.2.1 Barrier integrity

In order to validate the barrier integrity of the Caco-2 and EA.hy926 monolayers, TEER was measured before and after treatment. Prior to the treatment, all TEER values of the Caco-2 cells were above 1750 Ω*cm^2, indicating a good barrier integrity. The average ratios of TEER values after/before treatment are represented in Figure 16 and 17. For model A, it was observed that quercetin exposure to LPS-stressed Caco-2 cells improved the barrier integrity (Figure 16A), while an opposite trend was observed for the stressed EA.hy926 cells (Figure 16B). However, the observed effects were not greater than 20%. As a rule of thumb, 20% variation could be assigned to biological variation rather than a physiological effect. These results are thus statistical significant, but probably not biologically relevant. However, before stating conclusions, the experiment should be independently repeated several times. No trend in change of barrier integrity was observed for model B (Figure 17A, 17B).

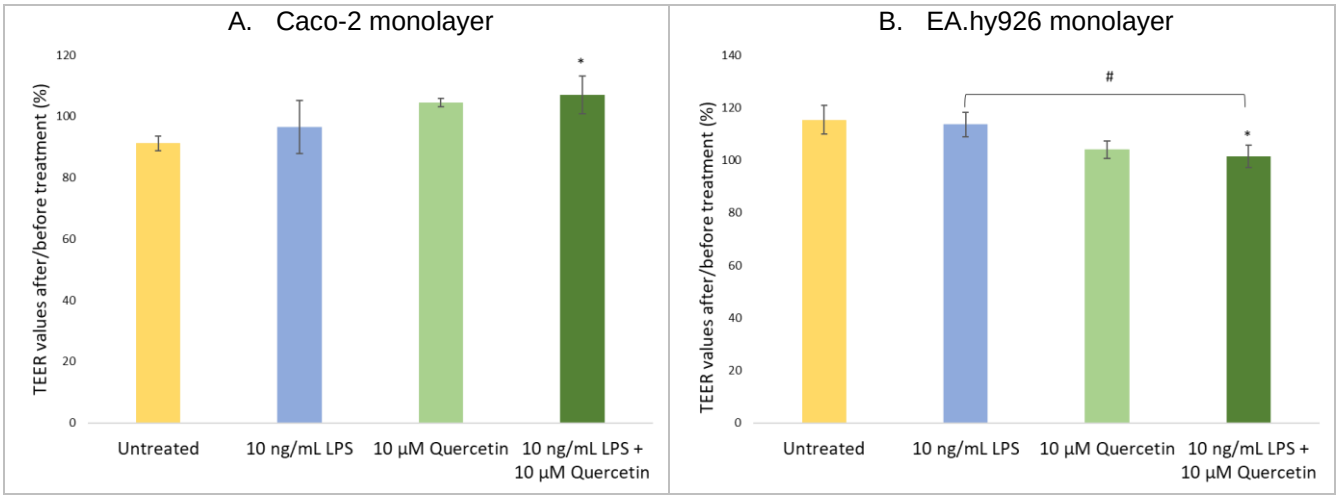


*Figure 16. The average ratios of TEER values after/before treatment in model A. The standard deviation is displayed by error bars. Significant differences with the reference (untreated cells) are indicated with * (p<0.05: *, p<0.01: **, p<0.001: ***). Significant differences between cells treated with and without quercetin are indicated with # (p<0.05: #, p<0.01: ##, p<0.001: ###).*

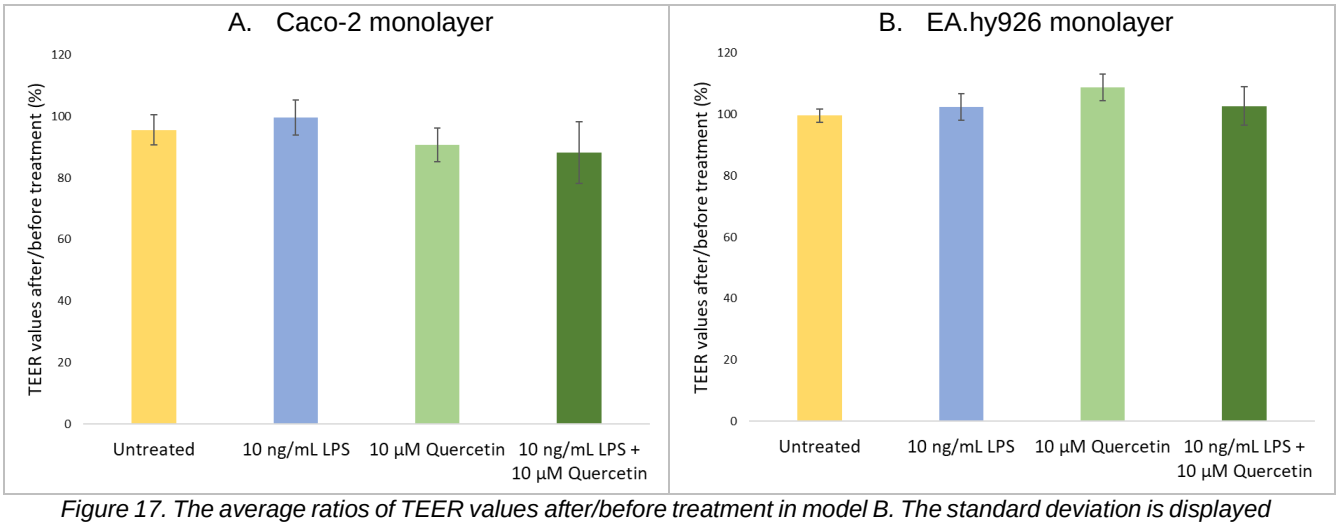


*Figure 17. The average ratios of TEER values after/before treatment in model B. The standard deviation is displayed by error bars. Significant differences with the reference (untreated cells) are indicated with * (p<0.05: *, p<0.01: **, p<0.001: ***). Significant differences between cells treated with and without quercetin are indicated with # (p<0.05: #, p<0.01: ##, p<0.001: ###).*

4.2.2 Quantification of monocyte adhesion

The degree of monocyte adhesion in model A was determined by counting the Calcein violet-AM-labelled THP-1 cells that had adhered to the EA.hy926 monolayer. A selection of microscopic images pertaining to model A and model B are given in Figure 18 and 19, respectively. The amount of THP-1 cells that could be detected on the filter membranes was too low to quantify. This can be explained by the force of gravity that is stronger than monocyte attraction by cytokines. Therefore, the focus is on the amount of THP-1 cells adherent to the basal endothelial monolayer. Figure 20 shows that the amount of adhered monocytes to the endothelium in model A tends to be higher for LPS-stressed endothelial cells, compared to untreated cells. Co-administration with quercetin could not counteract this effect. Figure 21 illustrates the results obtained by model B and shows that LPS stress indeed increase the monocyte adhesion. Moreover, quercetin co-administration counteracted the increased monocyte adhesion upon LPS stress. When comparing Figure 18 and 20 with Figure 19 and 21, respectively, it is observed that the density of fluorescent THP-1 cells is remarkably higher in the figures pertaining to model B (Figure 19, 21). In other words, more THP-1 cells have adhered to the basal endothelial monolayer in model B, compared to model A.

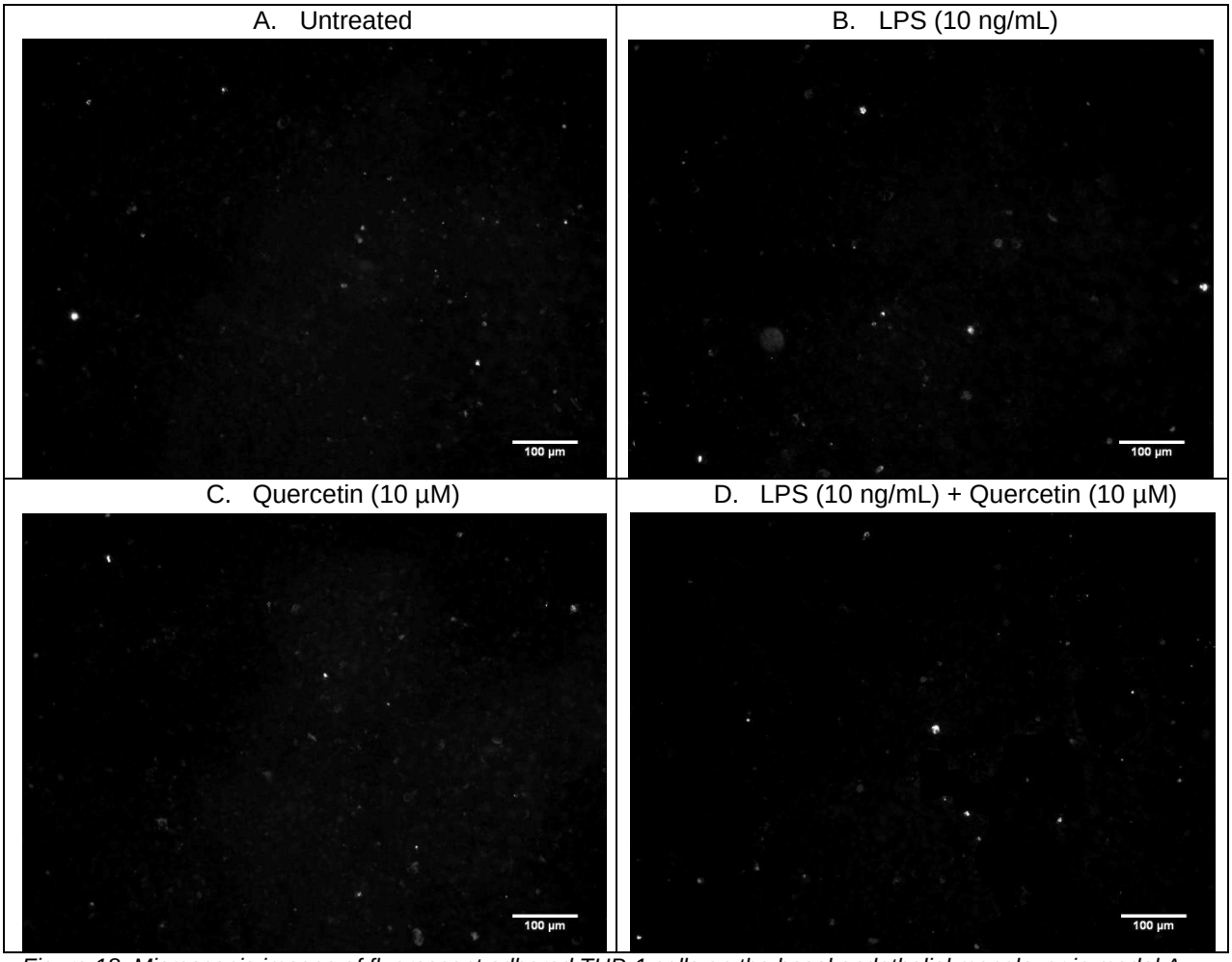


Figure 18. Microscopic images of fluorescent adhered THP-1 cells on the basal endothelial monolayer in model A. For each condition, 1 of the 9 images is presented.

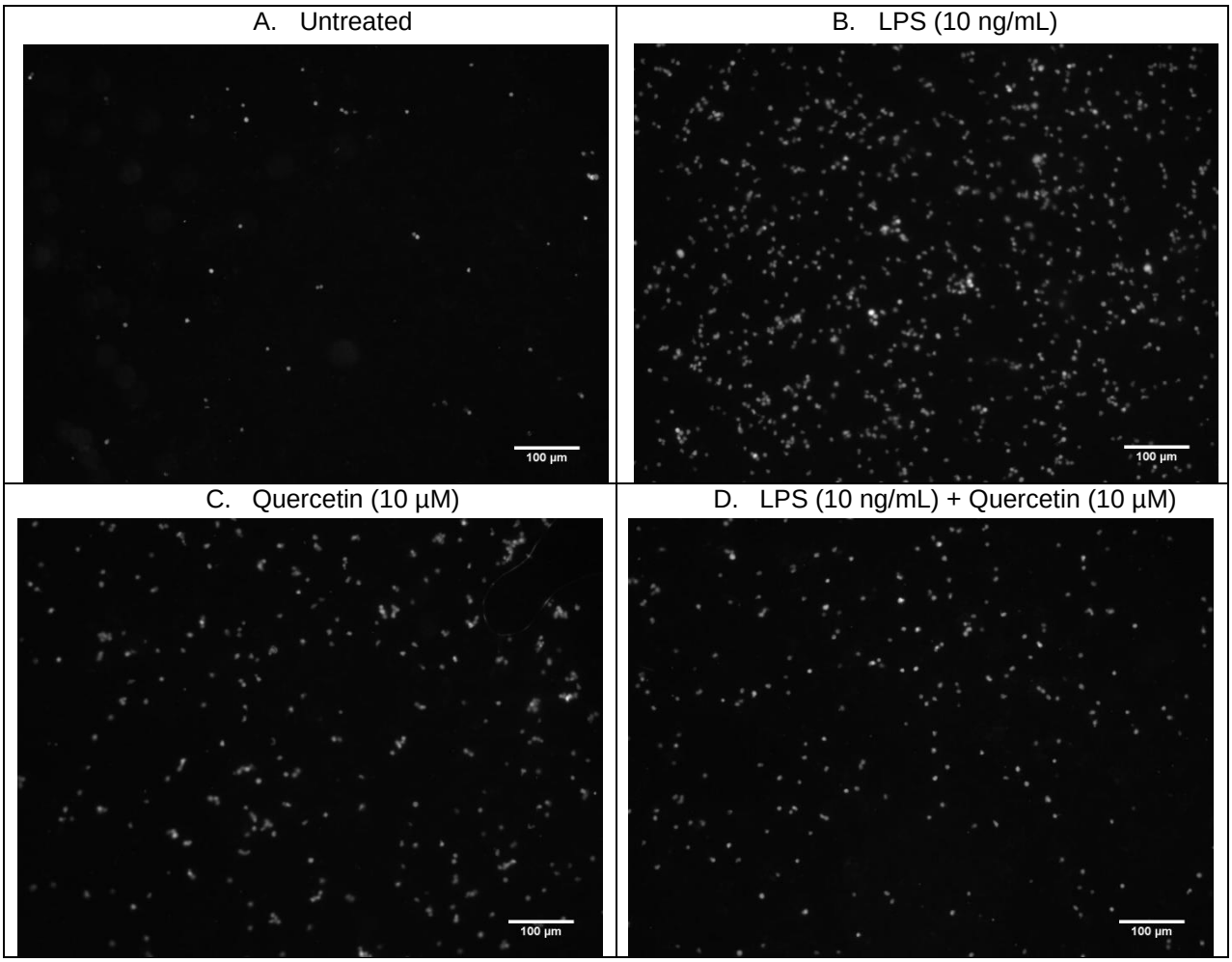


Figure 19. Microscopic images of fluorescent adhered THP-1 cells on the basal endothelial monolayer in model B. For each condition, 1 of the 9 images is presented.

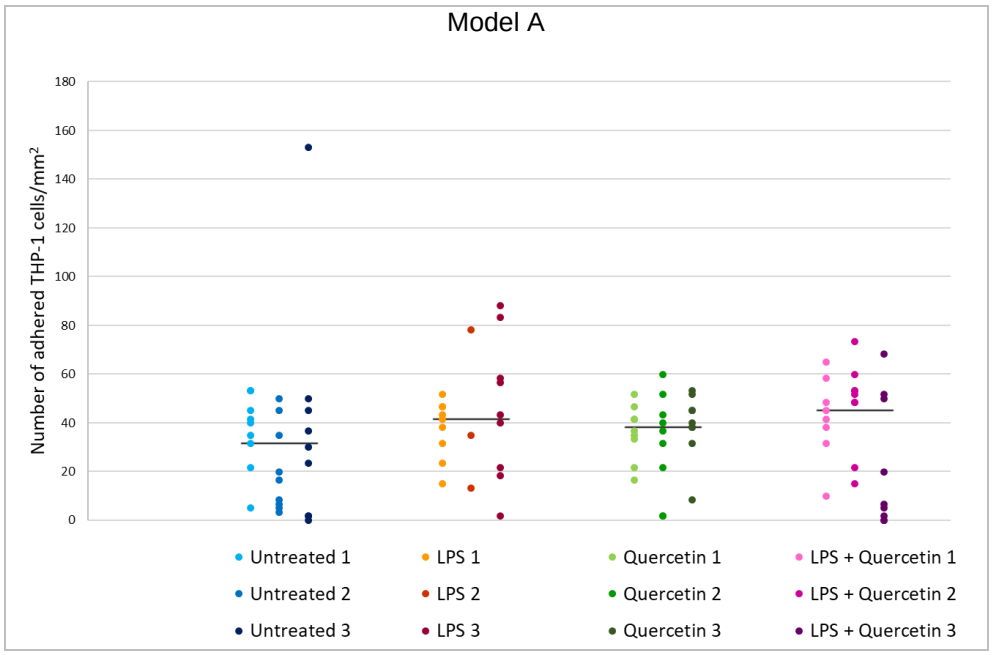


*Figure 20. Each dot represents the density of adhered THP-1 cells to the basal EA.hy926 monolayer on one microscopic image in model A. Every condition has three replicates (designated with a number and another colour). The median value of every condition is indicated with an horizontal line. Significant differences with the reference (untreated) are indicated with * (p<0.05: *, p<0.01: **, p<0.001: ***). Significant differences between conditions with and without quercetin are indicated with # (p<0.05: #, p<0.01: ##, p<0.001: ###).*

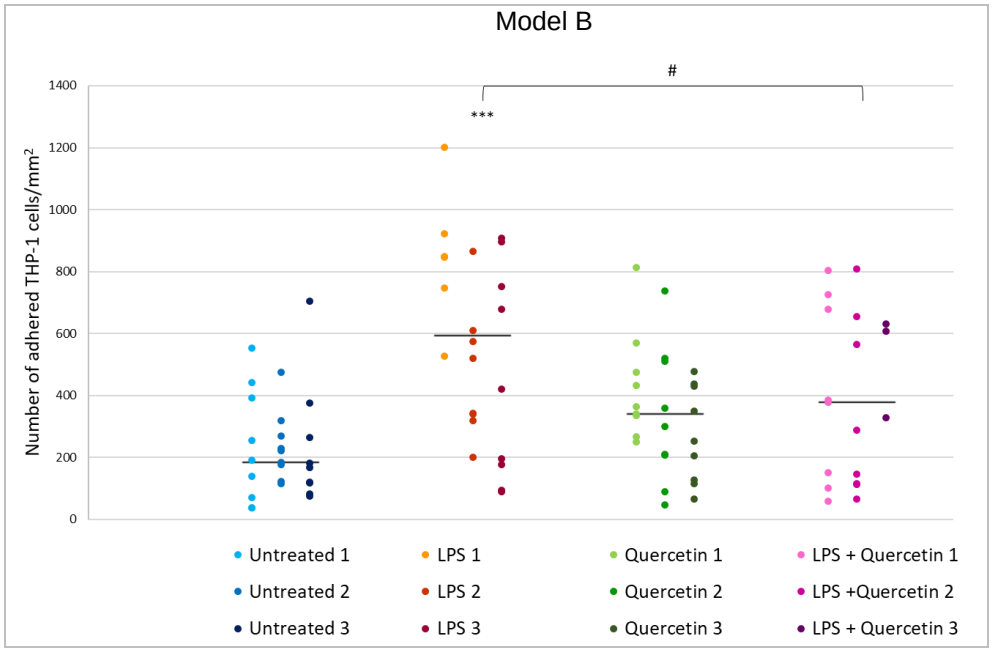


*Figure 21. Each dot represents the density of adhered THP-1 cells to the basal EA.hy926 monolayer on one microscopic image in model B. Every condition has three replicates (designated with a number and another colour). The median value of every condition is indicated with an horizontal line. Significant differences with the reference (untreated) are indicated with * (p<0.05: *, p<0.01: **, p<0.001: ***). Significant differences between conditions with and without quercetin are indicated with # (p<0.05: #, p<0.01: ##, p<0.001: ###).*

5. DISCUSSION

A main finding of this study is that quercetin accumulation in intestinal cells increased upon adrenaline stress and decreased upon cortisol stress. Moreover, novel triple co-culture models were developed to investigate the impact of quercetin on LPS-induced endothelial dysfunction. Using these *in vitro* models, it was observed that LPS induced endothelial dysfunction, thereby increasing monocyte adhesion to endothelial cells, whereas quercetin was able to counteract this effect.

5.1 Monocultures

The aim of this study was to investigate the effect of quercetin on *in vitro* cell culture models for endothelial dysfunction. As a first step, intracellular accumulation of quercetin was explored in monocultures of an intestinal (Caco-2) and endothelial (EA.hy926) cell line. Vissenaekens et al. (2019) reported an increase in quercetin accumulation in proliferating Caco-2 and EA.hy926 cells upon valinomycin stress and in proliferating Caco-2 cells upon LPS stress. There was no significant effect of TNF-α stress on quercetin accumulation. As a follow-up to these findings, intracellular quercetin accumulation upon stress induced by adrenaline and cortisol, two stress molecules that besides LPS and TNF-α can also be involved in the development of atherosclerosis, is examined in this study. Based on both *in vitro* and *in vivo* studies, concentration ranges of cortisol (10 nM – 1 µM for Caco-2 and EA.hy926) and adrenaline (1 µM – 20 µM for Caco-2 and 1 nM – 1 µM for EA.hy926) for application in the present quercetin accumulation study were determined. As described in the literature study of this thesis (section 1.1.5), psychological stress results in the release of adrenaline and cortisol in the blood. Adrenaline levels in normal resting patients are mostly below 0.2 nM and may increase by more than 10-fold during exercise (Berkin, Walker, Inglis, Ball, & Thomson, 1988; Buhler, da Prada, Haefely, & Picotti, 1978; Wortsman, Frank, & Cryer, 1984). Wortsman et al. (1984) reported that adrenaline levels in patients that endure chronic stress due to a severe illness or acute maximal stress after resuscitation following cardiac attack, can reach up to 7.5 nM and 196 nM, respectively. Cortisol levels in the morning fluctuate around 400 nM (Ljubijankic et al., 2008; Tabata, Atomi, & Miyashita, 1984; Turakitwanakan, Mekseepralard, & Busarakumtragul, 2013). An experiment of Tabata et al. (1984) with 10 patients reported that a mean cortisol level of 383 nM in the morning increased to 1024 nM on average after a 3h exercise. After performing a literature research, it is observed that several *in vitro* studies using Caco-2 or endothelial HUVEC cells, applied adrenaline concentrations (0-10 mM and 0-10 µM, respectively) that are much higher than physiological relevant (Carlson, Beiting, Kiani, Abell, & McGillis, 1996; El Moussawi et al., 2018; Kimoto et al., 2009; Martin, Liberati, & Diebel, 2017; Xiong et al., 2009b). In contrast, concentrations for cortisol applied to Caco-2 cells (500 nM) and HUVEC cells (0 – 1.2 µM) lean more towards physiological relevant values (Liu, Mladinov, Pietrusz, Usa, & Liang, 2009; Logie et al., 2010; Zheng et al., 2017).

By means of a resazurin assay, it was ensured that the applied adrenaline and cortisol concentration ranges in this study did not cause excessive cytotoxicity. However, a significant decrease in cell viability of Caco-2 cells upon adrenaline and cortisol stress was observed, while quercetin counteracted this effect. The significant increase in protein content in Caco-2 cells upon adrenaline stress is remarkable.

Adrenaline is reported to be involved in cancer cell survival and proliferation (Eng et al., 2014). Since undifferentiated Caco-2 cells may still have cancer cell properties, adrenaline could potentially exert the same effects on these cells. However, stress can also increase cellular protein levels (e.g. upregulation of heat-shock proteins), which could also be an elucidation for the observed results of the SRB assay (Malago et al., 2003). In literature, quercetin is described to be a potent inhibitor of heat-shock proteins, which is in accordance with the observed results as well (Arnal & Lalles, 2016).

The contact of adrenaline and cortisol with endothelial cells is straightforward, since these stress molecules circulate in the blood stream. However, adrenaline and cortisol interact with intestinal cells as well. Adrenaline is able to affect the gut motility and absorption rate of several nutrients in the gut. For example, adrenaline can increase glucose absorption by stimulation of β-adrenergic receptors, leading to the enhancement of SGLT1 transporter function (Ishikawa, Eguchi, & Ishida, 1997; Mittal et al., 2017). Moreover, α2-adrenergic receptors stimulate translocation of oligopeptide transporters to the apical membrane of mucosal intestinal cells, thereby increasing oligopeptide uptake (Berlioz et al., 2000). The uptake of ginsenoside Rg1 in Caco-2 cells was observed to be enhanced by the interaction of adrenaline with β2-receptors, thereby regulating SGLT1 transporters (Xiong et al., 2009a, 2009b). Interestingly, in this thesis it was observed that adrenaline significantly increased quercetin accumulation in Caco-2 cells at an optimal concentration of 1 µM. There may be a link with the increased nutrient absorption, although it must be noted that uptake of the quercetin aglycone occurs via passive diffusion or active transport through OATPs. Upregulation of influx transporter molecules such as SGLT1 by adrenaline could have an impact on the absorption of quercetin glucosides, since uptake of the latter occurs via this transporter but probably not on the aglycone uptake. Considering that passive diffusion only augments upon an increasing concentration of extracellular quercetin, it can be suggested that an increase in quercetin accumulation upon adrenaline stress is induced by an increase in OATPs expression/activity or decrease in the expression/activity of efflux transporters. As described in the literature study of this thesis (section 1.1.5), stimulation of β-adrenergic receptors by adrenaline leads to adenylyl cyclase activation and an increase in intracellular cAMP concentrations. The MRP4 transporter is reported to regulate cAMP homeostasis by extruding cAMP from the cells. Since MRP4 is also a known efflux transporter for quercetin (metabolites), the latter can compete with cAMP molecules for these transporters, resulting in a diminished efflux and increased accumulation of quercetin (metabolites) (Sassi et al., 2012; M. Xie, Rich, Scheitrum, Conti, & Richter, 2011). Furthermore, adrenaline showed to decrease MRP2 and MRP3 mRNA expression in primary liver cells, which may also be the case in Caco-2 cells (Mayati et al., 2017).

When assuming that the observed adrenaline effect is caused by binding of adrenaline to one of its receptors, the presence of at least some adrenergic receptors in Caco-2 cells can be suggested. Although controversy exists about the presence and localisation of these receptors, they will most likely be located at the basolateral membrane (Valet et al., 1993). However, since proliferating and thus non-polarized Caco-2 cells were used for the quercetin accumulation assay, the localisation aspect will presumably not have an impact on the obtained results.

Similar to adrenaline, cortisol interacts with intestinal cells as well. As described in the literature study of this thesis (section 1.1.5), cortisol mobilizes peripheral glucose e.g. by promoting gluconeogenesis in the liver. However, after ingesting a glucose-rich meal, blood glucose levels increase and subsequent insulin release stimulates peripheral glucose uptake and attenuates hepatic gluconeogenesis. Since this is antagonizing with the cortisol operation, cortisol is capable of inhibiting glucose absorption in the intestine. The decrease in translocation of the GLUT2 glucose transporters to the apical membrane of intestinal cells in response to cortisol is described in literature (Shepherd et al., 2004). In contrast to adrenaline, cortisol was not observed to increase quercetin accumulation in Caco-2 cells. Interestingly, a decreasing trend in quercetin accumulation upon increasing cortisol stress could be observed. There could be a link between quercetin accumulation in Caco-2 cells and glucose absorption in the intestine, however this would be unlikely, since Kwon et al. (2007) demonstrated that the quercetin aglycone was not transported through GLUT2, GLUT5 and SGLT1 in oocytes. Moreover, quercetin showed to non-competitively inhibit the intestinal GLUT2 transporter. It is hypothesized that cortisol dose-dependently hinders quercetin uptake or enhances quercetin efflux in Caco-2 cells. Interestingly, both cortisol and quercetin efflux can occur through P-glycoprotein (van Kalken et al., 1993). Competition for this efflux transporter would imply an expected increase in quercetin accumulation, which is contradictory with the observed results. Therefore, another mechanism is proposed as possible elucidation. Analogous to the decreased translocation of glucose transporters, cortisol could potentially enhance the translocation of efflux transporters such as MRPs, thereby increasing quercetin efflux. More research needs to be conducted in order to unravel the mechanistic pathways behind the observed effects of adrenaline and cortisol. Moreover, it should be noted that the observed effects are based on the results of one experiment, so multiple independent repetitions are recommended in order to confirm the conclusions.

As discussed in the literature study of this thesis (section 1.2.3), quercetin is first transported and transformed by intestinal cells before reaching peripheral cells through the blood. Due to the low quercetin bioavailability, endothelial cells are exposed to a much lower quercetin concentration than the intestinal cells. Therefore, based on the quercetin accumulation experiments by Vissenaekens et al. (2019), Caco-2 and EA.hy926 cells were exposed to 10 µM and 3 µM quercetin, respectively. After performing the accumulation assay on EA.hy926 with 10 µM quercetin, relatively low fluorescent signals for intracellular quercetin accumulation were observed (results not shown), suggesting that quercetin accumulated less in endothelial cells, compared to intestinal cells. The lower amount of administered quercetin (3 µM) combined with a smaller quercetin accumulation can result in a very weak fluorescent signal. After correction of fluorescent background, small values with large variations were obtained. The large variability impedes correct interpretation of the results, hence no reliable conclusions can be drawn about the effect of adrenaline and cortisol on the accumulation of quercetin in endothelial cells.

From the quercetin accumulation experiments, where ascorbic acid was supplemented to the medium, it could not be concluded that the latter counteracted potential degradation of quercetin. Bandaruk et al. (2014) described the instability of quercetin in culture medium. However, in the experiment performed in this thesis, intracellular quercetin was measured. As reported in literature, quercetin is able to

accumulate in organelles such as mitochondria or bind to intracellular proteins (Fiorani et al., 2010; Kaldas, Walle, van der Woude, McMillan, & Walle, 2005). Bound quercetin can be presumed to be more stable and thus less prone to degradation than quercetin in cell culture medium. The low degree of quercetin degradation could be a possible elucidation for the seemingly lacking effect of ascorbic acid.
It should be noted that there is an inter-day and -plate variability for the results of the accumulation study with and without ascorbic acid, which impedes drawing reliable conclusions about the real biological effect of ascorbic acid on quercetin accumulation in stressed cells. Therefore, additional accumulation assays should be performed with and without ascorbic acid in the same 96-well plate in order to eliminate inter-day and -plate variability.

5.2 Triple co-cultures

Three triple co-culture models for endothelial dysfunction containing intestinal, endothelial cells and monocytes were developed. As illustrated in Figure 9 in section 3.3.2, each model is subdivided in three compartments representing different compartments in the body. In model A and B, compartment 1 reflects the intestinal lumen, whereas compartments 2 and 3 represent the interstitial space and the vascular lumen, respectively. As described in materials and methods (section 3.3.2), the composition of the growth medium differs in each compartment. Evidently, the glucose concentration in the intestinal lumen exceeds the glucose concentration in the blood. According to Ferraris et al. (1990), intestinal luminal glucose concentrations range between 36 mg/L (0.2 mM) and 9 g/L (48 mM) under normal conditions. Based on this, cell culture medium containing 4.5 g/L (25 mM) glucose was applied to compartment 1. Normal blood glucose concentrations range between 0.8 g/L (4.4 mM) and 1.2 g/L (6.7 mM) (McMillin, 1990), therefore, cell culture medium containing 1 g/L (5.6 mM) glucose was administered to compartments 2 and 3. Additionally, the cell culture medium of compartment 3 in model B comprised 10% (v/v) FBS, since serum is an important compound in the blood, but is not physiological relevant in the intestinal luminal or interstitial space. Due to the experimental set-up of model A, serum could possibly cause interference in the ELISA assay, therefore FBS was not supplemented to compartment 3 in model A. Analogously, there was no FBS administered to compartment 3 in model B during after-treatment.
In order to obtain a model for endothelial dysfunction, endothelial cells were stressed with LPS. As described in the literature study of this thesis (section 1.4.3), physiological relevant concentrations of LPS range between 0 and 1 ng/mL and can reach up to 10 ng/mL in diseased conditions, whereas concentrations applied *in vitro* usually exceed this range. In order to induce endothelial dysfunction, a LPS concentration of 10 ng/mL was applied. The biotransformation and low bioavailability of quercetin was taken into account in the co-culture models by including an intestinal barrier to which 10 µM quercetin was apically administrated.

As described in the literature study of this thesis, several studies have reported the inhibitory effect of quercetin on LPS-induced upregulation of pro-inflammatory cytokines, adhesion molecules in endothelial cells and subsequent monocyte adhesion. Therefore, in the three triple co-culture models, an observed increase in adhered monocytes to LPS-exposed endothelial cells and a counteracting effect

of quercetin is hypothesized. A mechanistic pathway behind this hypothesis can be extracted from literature, and was taken as a basis to construct the scheme in Figure 22. Upon LPS stimulation of the TLR4-CD14-MD2 receptor complex in endothelial cells, homodimerization of the TLR4 receptors takes place, resulting in the recruitment of several signalling molecules including myeloid differentiation factor 88 (MyD88). This leads to the activation of the IκB kinase (IKK) complex which phosphorylates IκB, an inhibitory protein of NF-κB, causing its degradation. After release from IκB in the cytoplasm, NF-κB is translocated to the nucleus where it can act as a transcription factor for i.a. pro-inflammatory genes. IKK can also be activated via the phosphatidylinositol 3' kinase (PI3K)/Akt pathway (Dauphinee & Karsan, 2006).

Besides NF-κB, activator protein 1 (AP-1) is another crucial transcription factor capable of regulating the expression of pro-inflammatory and adhesion molecules. LPS stimulation can induce a mitogen activated protein kinase (MAPK) cascade, resulting in the activation of AP-1 (Morioka et al., 2012; W. S. Yang et al., 2013). Furthermore, a MyD88-independent pathway is described, involving the recruitment of signalling molecules such as adapter-inducing interferon-β (TRIF), leading to IKK-mediated NF-κB activation (Dauphinee & Karsan, 2006; Y. C. Lu, Yeh, & Ohashi, 2008).

The counteracting effect of quercetin can be assigned to several mechanisms. For example, quercetin has the ability to enhance nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated transcription of anti-inflammatory and anti-oxidant genes such as HO-1 and NAD(P)H quinone 1 dehydrogenase (NQO1). For instance, NQO1 protects cells against oxidative stress, whereas HO-1 additionally inhibits AP-1 activity and adhesion molecule expression (C. Li, Zhang, & Frei, 2016). While oxidative stress can both activate and inhibit NF-κB activation, it can also induce the Nrf2 pathway as an anti-oxidant defense. As illustrated in Figure 22, NF-κB and Nrf2 have an antagonizing effect on each other (Ahmed, Luo, Namani, Wang, & Tang, 2017; Lingappan, 2018). Nrf2 is sequestered in the cytoplasm by association with Kelch-like ECH-associated protein 1 (Keap1). The latter mediates Nrf2 ubiquitination and subsequent proteasomal degradation. Tanigawa et al. (2007) observed that quercetin increased the Nrf2/Keap1 ratio in HepG2 liver cells in several ways. First of all, quercetin enhanced Nrf2 transcription and thus Nrf2 mRNA levels. Second, as a result of quercetin activity, Nrf2 was stabilized by inhibition of its ubiquitination. Although quercetin did not provoke Nrf2-Keap1 dissociation, a modified Keap1 protein was formed, leading to the reduction of Keap1 steady-state levels through proteasome-independent degradation. Finally, the increased Nfr2/Keap1 ratio implies a reduction in Nrf2-Keap associations, whereby Nrf2 ubiquitination is decreased and nuclear translocation of Nrf2 is enhanced, hence forming a positive feedback loop (Tanigawa et al., 2007). It should be noted that this study was performed in HepG2 cells. No studies regarding the described mechanism in endothelial cells are available in literature. The attenuation of NF-κB activity by quercetin in HUVEC cells was described by Cho et al. (2016). Furthermore, Bian et al. (2018) reported that quercetin has the ability to counteract the LPS-mediated upregulation of the protein expression of the NF-κB p65 subunit, TLR4, MyD88 and downregulation of IκB in rat intestinal microvascular endothelial cells (RIMVECs). In addition, quercetin reduced the LPS-induced p65 and JNK – i.e. a MAPK – activation in RIMVECs (Bian et al., 2018). Moreover, quercetin showed to attenuate the LPS-induced upregulation of phosphorylated Akt, JNK and

IKK levels in RAW264.7 macrophages (Chang, Tsai, Sheu, Hsieh, & Chiang, 2013). Based on these reports, it could be suggested that quercetin also exhibits an inhibitory effect on the protein expression and activation of several mediators in the LPS-induced pro-inflammatory pathway. However, it should be noted that the pathways induced by LPS and quercetin are not that straightforward. For example, the PI3K/Akt pathway can also be involved in the activation of Nrf2 (G. Chen et al., 2014). Furthermore, there are several studies that reported the involvement of MAPKs in Nrf2 regulation. However, the exact mechanisms are not well understood yet. Results regarding the effect of MAPKs on Nrf2 activation are contradictory (Z. Sun, Huang, & Zhang, 2009). While some studies suggest that quercetin induces Nrf2 via p38 activation, other studies demonstrate that inhibition of p38 stimulates Nrf2 induction (Granado-Serrano, Martin, Bravo, Goya, & Ramos, 2012; C. Li et al., 2016; Naidu, Vijayan, Santoso, Kietzmann, & Immenschuh, 2009). More research should be performed towards the mechanisms and crosstalk between several pathways.

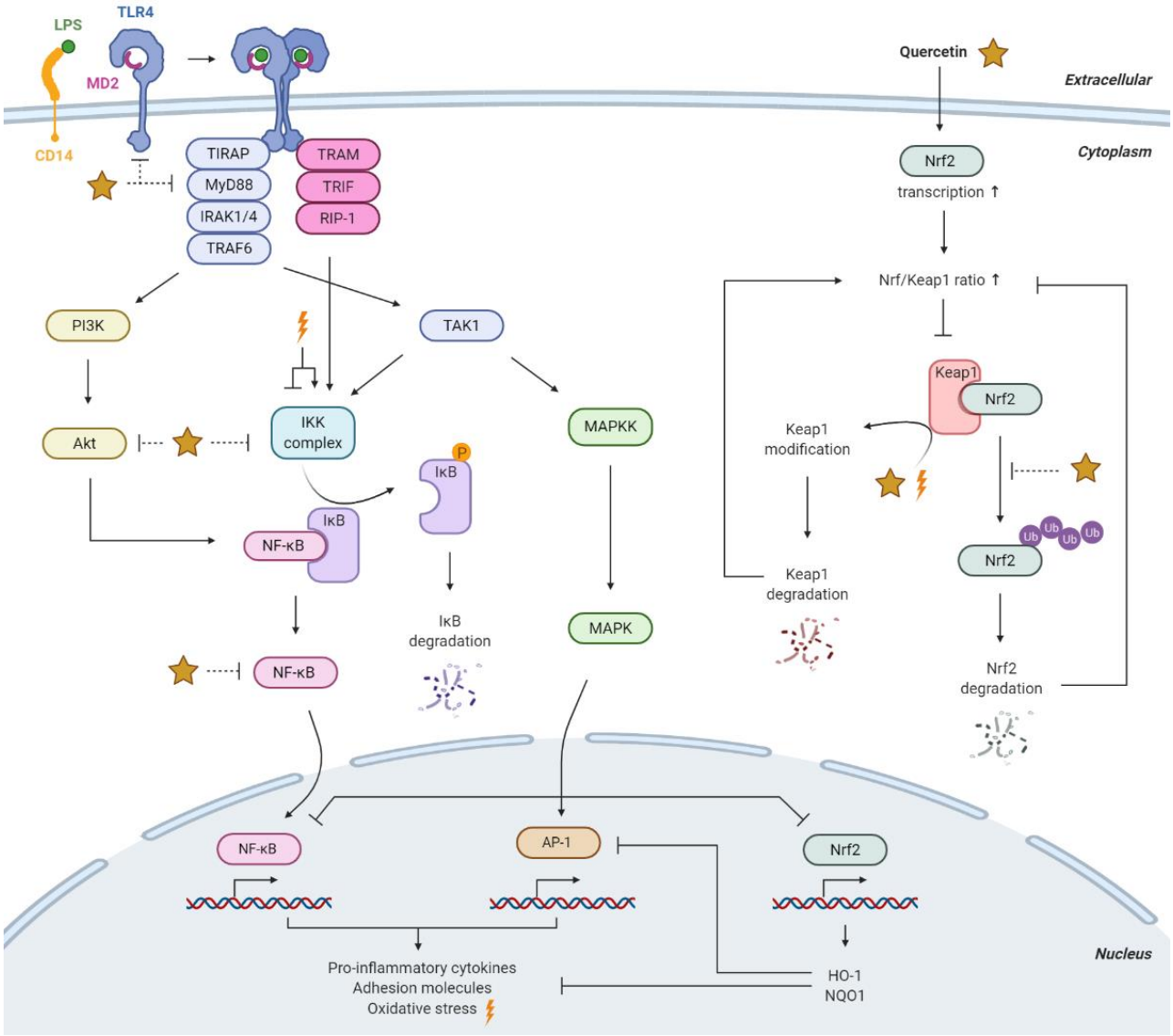


Figure 22. Proposed mechanistic pathway behind the LPS-induced oxidative stress (⚡) and upregulation of pro-inflammatory and adhesion molecules, and the counteracting effect of quercetin in endothelial cells. The dashed lines (---|) indicate a suggested inhibitory effect of quercetin (★).

Using model B, where the monocytes are present in compartment 3 for 4h, it was indeed found that monocytes adhered more to the LPS-stressed endothelium. As hypothesized above, this was counteracted by co-administration of quercetin. Using model A, where the monocytes are present in compartment 3 for 24h, this protective effect of quercetin co-administration was not significant, moreover, the total amount of adhered monocytes was much lower compared to the set-up using model B. Based on the findings of Al-Numani et al. (2003), adhesion molecule (ICAM-1 and LFA-1) expression in THP-1 monocytes after LPS stimulation is expected to increase over a time range of minimal 24 hours. Moreover, the expression levels of VCAM-1 in HUVEC cells still increased 24 hours after LPS stimulation and although the expression levels of E-selectin and ICAM-1 moderately decreased, they were not returned to baseline levels yet (Al-Numani et al., 2003). A higher expression of adhesion

molecules would be expected to be correlated with a higher degree of monocyte adhesion. However, since the ELISA assay could not be performed due to the Covid-19 measures, no conclusions can be drawn here regarding the level of cytokines and adhesion molecules in relation to monocyte adhesion. Moreover, model A and B were developed several months apart from each other, introducing a lot of variability which can impede comparison. In case the results of the ELISA assay would concord with the observations of Al-Numani et al. (2003), a few possible explanations for the results of the monocyte adhesion assay could be suggested. Since the THP-1 cells are present in the basal compartment for 24 hours in model A, these cells could already have migrated across the endothelium, thereby not being visible on the endothelial monolayer anymore. Although, since the basal endothelial monolayer resides on a glass slide instead of a filter membrane, it is doubtful that the monocytes can penetrate under the endothelial cells that are attached to the glass slide. Another elucidation could be the spontaneous detachment of monocytes from the endothelial monolayer after several hours – as described for lymphocytes by de Bono (1976) – and their release back into the medium. Evidently, these monocytes are then removed after the washing step. This could also explain the observation of a higher density of adhered monocytes in model B compared to model A.

The large variation in monocytes density between the different microscopic images belonging to same replicate is remarkable. Due to certain actions such as the washing step and manipulations with the glass slide for microscopic imaging, residual loose monocytes may accumulate in particular regions, causing a heterogenous density. By using an immunohistochemical stain, THP-1 cells could be non-fluorescently marked, enabling imaging of the whole glass slide and subsequent counting of the total amount adhered monocytes. Furthermore, determination of the LOD and LOQ, using microscopic images of an endothelial monolayer without fluorescent THP-1 cells, can be suggested. This can be especially of value for the images of model A, since the observed fluorescent signals in each image were rather limited in number. If these numbers turn out to be below LOD and LOQ, no conclusions can be drawn regarding the presence and the number of adhered monocytes, respectively.

As described in the literature study of this thesis (section 1.2.3), quercetin is extensively metabolized in the liver, although metabolizing enzymes are also present in intestinal cells, suggesting that quercetin metabolites can also be formed here. Indeed, (conjugated) quercetin and (conjugated) isorhamnetin (3'-O-methyl-quercetin) were found intracellular and in the basolateral solution after apical quercetin aglycone (10 µM) administration to differentiated Caco-2 cells (Murota, Shimizu, Chujo, Moon, & Terao, 2000). Similarly, Gonzales et al. (2016) observed that a small amount of quercetin aglycone and methylated quercetin was retained in undifferentiated Caco-2 cells, while the quercetin aglycone, along with methyl, sulfate and glucuronide conjugates were detected in the cell culture medium. Methylated derivatives were observed to be predominant. Moreover, an increase in (methylated) quercetin accumulation in Caco-2 cells was observed upon valinomycin stress (Gonzales et al., 2016). However, in a study by Vissenaekens et al. (2019), LPS did not increase quercetin accumulation in quiescent endothelial cells, so an enhanced accumulation due to LPS stress cannot be assumed for the triple co-culture models in this thesis. Still, quercetin aglycone accumulation could be increased by deconjugation

of quercetin glucuronides at vascular level, rendering the aglycone and its subsequent uptake in cells (Perez-Vizcaino, Duarte, & Santos-Buelga, 2012).

Lotito et al. (2011) reported a significant change in bioactivity upon quercetin modification. Whereas quercetin, 3'-O-methyl quercetin and 4'-O-methyl quercetin showed to reduce ICAM-1 and E-selectin expression in TNF-α stressed HAECs, quercetin-3-O-glucuronide and quercetin 3'-O-sulfate were not effective. The most significant effect was observed with quercetin aglycone (Lotito et al., 2011). It should be noted that not all quercetin metabolites were tested, e.g. quercetin-methylglucuronides and quercetin-methylsulfates, so one has to be careful with drawing conclusions about the specific effect of sulfation, glucuronidation or methylation on quercetin bioactivity. Based on the aforementioned studies, following assumptions can be made: (i) both quercetin aglycone as methyl derivatives are presumed to attenuate monocyte adhesion in triple co-culture model B, (ii) of the quercetin metabolites extruded by Caco-2 cells, methyl derivatives are most abundant, although they have potentially a lower bioactivity towards adhesion molecule expression than the aglycone, (iii) quercetin aglycone is not abundantly extruded by Caco-2 cells, although its levels can be augmented by deconjugation of quercetin glucuronides at endothelial level, thereby increasing quercetin aglycone accumulation and its biological effect. However, in order to be able to confirm these assumptions, identification and quantification of quercetin metabolites in the different compartments and in the endothelial cells in the triple co-cultures is required.

The main advantage of the triple co-culture models developed in this thesis is the incorporation of the intestinal, vascular and immune system in one *in vitro* model. The crosstalk between endothelial cells and monocytes is an important event in the pathogenesis of atherosclerosis. This is already explored using endothelial cell - monocyte co-cultures, as described in the literature study of this thesis (section 1.4.2.2). However, when investigating the beneficial effect of polyphenols *in vitro*, it is crucial to take the absorption and metabolism into account. Therefore, an intestinal epithelial barrier was included in this model, resulting in the exposure of endothelial cells to a lower amount of quercetin (metabolites), thereby increasing the physiological relevance. In spite of the involvement of three biological systems in one model, it should be noted that this is still a very simplified representation that deviates extensively from the *in vivo* situation. Several factors are not included in this model. First of all, as described in section 1.2, quercetin is mainly found in plant-based food products as glycoside derivatives. Quercetin glycosides containing a rhamnose unit are first metabolized by microbiota in the large intestine before absorption. Moreover, flavonoids that are not absorbed in the small intestine or flavonoid metabolites that are excreted from the bile back into the small intestine, can be further metabolized by microbiota in the colon as well. The metabolizing role of microbiota is a lacking aspect in this model, although this model is still useful for the absorption and metabolism of quercetin aglycone in the small intestine. Second, each biological system is represented by only one cell type. For example, the intestinal barrier is represented by a monolayer of intestinal absorptive Caco-2 cells, since these cells form the greatest barrier for nutrient passage. However, mucus contributes to the barrier integrity as well. Therefore, a co-culture of mucus-secreting HT29-cells and Caco-2 cells as described by Antunes et al. (2013) could be integrated in this model. Although Caco-2 cells contain the same metabolizing enzymes as hepatocytes,

polyphenol metabolism occurs mainly in the liver which is also a missing factor in this model. Monocytes play a crucial role in the onset and progression – by differentiation into macrophages – of atherosclerosis. Therefore, these cells were chosen to be used in the experimental part of this thesis, more specifically to investigate monocyte adhesion. However, it must be emphasized that the immune system is a very complex organization with a wide range of cells that are also involved in the pathogenesis of atherosclerosis, such as neutrophils, lymphocytes and platelets. Noonan et al. (2019) developed a triple co-culture model for atherosclerosis including endothelial cells, monocytes and smooth muscle cells. Since the latter contribute to the progression of atherosclerosis, incorporation of these cells would be a step forward in the development of an atherosclerotic model.

6. CONCLUSION

In this study, the bioavailability of quercetin was investigated using monocultures stressed with psychological stress molecules, adrenaline and cortisol. It was found that intracellular quercetin accumulation was increased upon adrenaline stress in Caco-2 cells, whereas the opposite effect was seen upon cortisol exposure. It is suggested that these stressors act on mechanistic pathways regulating the influx or efflux of quercetin. More research should be conducted in order to unravel the mechanisms of action of adrenaline and cortisol in intestinal epithelial cells.

Bioactivity of quercetin was explored in novel triple co-culture models for endothelial dysfunction including intestinal cells, endothelial cells and monocytes. LPS was applied as a stressor to induce endothelial dysfunction. The intestinal monolayer in the model set-up is crucial to take bioavailability and biotransformation of quercetin into account. As expected, LPS increased monocyte adhesion to endothelial cells, whereas quercetin (metabolites) were able to counteract this effect. Identification and quantification of the quercetin metabolites, in the cell culture medium and intracellular, is required in order to make a distinction between the bioactivity of quercetin and its metabolites.

It should be noted that the set-up of the triple co-culture model is important as it can influence the observed results. For example, there was no significant difference between the degree of monocyte adhesion after different treatments (untreated, LPS, quercetin, LPS + quercetin) in a model where monocytes were administered before treatment (24h), in contrast to a model where monocytes were administered after treatment, followed by a 4h-later monocyte adhesion assay. The second model seems more appropriate for investigating the effect of quercetin (metabolites) on endothelial dysfunction. This novel triple co-culture model could have the potential to uncover the mechanistic pathway behind the *in vivo* observed beneficial effects of quercetin intake on atherosclerosis.

7. FUTURE PERSPECTIVES

Due to Covid-19 measures, some experiments could not be performed. The hypotheses regarding these experiments are described in this section.

First of all, samples for an ELISA assay were taken in each compartment of the triple co-culture models. The aim of the ELISA assay is to detect and quantify endothelial dysfunction biomarkers such as sICAM-1, sVCAM-1 and sE-selectin and pro-inflammatory cytokines such as TNF-α, IL-6, IL-8 and IL-1β. Since LPS is known to induce endothelial dysfunction and a pro-inflammatory state by upregulating the expression of adhesion and pro-inflammatory molecules, an increased concentration of these molecules is expected in compartment 3 of the LPS-treated wells. Since LPS is only administrated to compartment 3, a gradient in concentration of these biomarkers from compartment 3 to 1 can be expected. Based on the findings of several studies described in the literature study of this thesis (section 1.4.2) and the observed counteracting effect of quercetin on monocyte adhesion in model B, a diminished amount of pro-inflammatory and adhesion molecules is expected in compartment 3 of the LPS+quercetin-treated wells. Although the degree of monocyte adhesion in model A was rather low compared to model B, a correlation with the results of the ELISA assay is not guaranteed. As described in the discussion (section 5.2), the small amount of adhered monocytes in model A is assumed to be the result of monocyte extravasation or spontaneous detachment, rather than a decrease in cytokine/adhesion molecule levels. As observed by Chanput et al. (2010), only the levels of TNF-α secreted by LPS-stimulated THP-1 monocytes declined after 6 hours, whereas the levels of IL-8, IL-6 and IL-1β increased or stagnated during 30 hours after LPS stimulation. After 1 hour, IL-8 was predominant present in the extracellular medium, so this cytokine is expected to be highly detected using ELISA. Since the THP-1 monocytes in model B were only for 4 hours exposed to LPS, the amount of detected cytokines may even be lower than in model A. Moreover, the expression of adhesion molecules in THP-1 monocytes was observed to be higher 24 hours compared to 4 hours after LPS exposure (Al-Numani et al., 2003).

A third triple co-culture model was developed (model C), as illustrated in Figure 9. In this model, THP-1 monocytes are seeded at a density of 200 000 cells/mL on the apical side of a 6-well Transwell® membrane where they are differentiated into macrophages by the exposure to 500 nM PMA for 3 hours. Analogous to model A and B, the Caco-2 monolayer and basal EA.hy926 monolayer are grown in compartment 1 and 3, respectively. Moreover, the growth media in the different compartments and treatment steps are similar to model A, as displayed in Table 7 and 8. The goal of model C is to investigate the effect of LPS and quercetin on the inflammatory response of macrophages. In an early stage of atherosclerosis, monocytes are attracted to the activated endothelial wall, a process that is explored by model A and B. After extravasation, monocytes differentiate into macrophages. The latter produce inflammatory cytokines such as IL-1, IL-6, TNF-α and IFN-γ. The aim is to detect and quantify these molecules using an ELISA assay. Based on the findings of studies described in section 1.4.2 of the literature study, it is hypothesized that quercetin inhibits the LPS-induced increase in inflammatory cytokine levels. The effects of quercetin and LPS will presumably be seen in compartment 2 and 3 due to the presence of macrophages and endothelial cells, respectively. Furthermore, it is hypothesized that

a greater increase in inflammatory cytokines secretion will be seen in model C compared to model A and B. This hypothesis is based on the study by Chanput et al. (2010) in which a higher inflammatory cytokine secretion by THP-1 macrophages after LPS stimulation was observed compared to THP-1 monocytes. This finding could be explained by the upregulation of TLR4 receptors in PMA-treated THP-1 cells, enabling a higher LPS response (Zarember & Godowski, 2002).

In the three developed co-culture models, endothelial stress is induced by LPS. In future studies, it would be interesting to explore the effects of other stressors using these models. As described in the literature study (section 1.1.2), several stimuli are capable of activating endothelial cells by inducing a NF-κB-pathway. For example, TNF-α could be applied to simulate chronic inflammation, LDL for hypercholesterolemia and adrenaline or cortisol for psychological stress.

Over the last years, the use of co-culture models has become more frequent in research. The implementation of different cell types in one model has some advantages compared to monoculture models. For example, co-culture models allow crosstalk between different cell types, which is an event that also occurs *in vivo* and can have a significant impact on the results of the study. Moreover, due to the inclusion of an extra cell type in the model, certain important *in vivo* processes, such as nutrient absorption and metabolism by intestinal cells, are taken into account as well, rendering a more physiological relevant model. However, the development of co-culture models can also be challenging, since culture conditions of the different cell types can differ. In addition, it is more costly and time- and effort-consuming. Overall, the use of *in vitro* co-culture models may give new perspectives for more advanced research towards the mechanistic pathways behind the bioavailability and bioactivity of active compounds, not only in the context of flavonoids and atherosclerosis, but also more generally in the search for potential therapeutics for diseases. Nevertheless, the development of static 2D cultures for unraveling *in vivo* mechanisms is still limiting, since the micro-environment of static 2D cultures fails to simulate the *in vivo* situation. Hence, a switch to dynamic 2D cultures or 3D cultures could potentially be a better approach to mimic the *in vivo* environment (Edmondson, Broglie, Adcock, & Yang, 2014; Hansmann, Egger, & Kasper, 2018).

The importance of metabolite identification and quantification is already emphasized in the discussion (section 5.2). Therefore, it is strongly recommended to determine quercetin (metabolite) levels in the culture medium and endothelial cells by using analytical methods such as LC-MS/MS (Xiao, Zhou, & Ressom, 2012). In contrast to spectrofluorimetric methods, LC-MS/MS allows identification of quercetin metabolites, although it is more effort- and time-consuming. Identification of quercetin (metabolites) is required in order to uncover the bioactive compound(s).

As already demonstrated in other studies and confirmed by this study, bioactives can have an effect on stress molecules, but also the other way around. This suggests that health status is indeed a crucial factor in the research towards bioavailability and bioactivity of food nutrients. Therefore, stratification of the population based on health status in future clinical trials and epidemiological studies is strongly recommended, since this can reduce inter-individual variability within a diseased/healthy group. This way, bioavailability and bioactivity of food nutrients in a specific subpopulation can be determined more accurately, thereby enabling the recommendation of certain bioactives for these subpopulations.

Table 7. An overview of the growth media applied to the different compartments of model C during (pre-)treatment

	Pre-treatment (1h)		**Treatment (24h)**	
Compartment	4,5 g/L glucose DMEM supplemented with 1% (v/v) NEAA	1 g/L glucose DMEM	4,5 g/L glucose DMEM supplemented with 1% (v/v) NEAA	1 g/L glucose DMEM
Compartment 1	✓	×	✓	×
Compartment 2	×	✓	×	✓
Compartment 3	×	✓	×	✓

Table 8. An overview of quercetin and LPS administration to compartment 1 and 3 of model C during (pre-)treatment

	Pre-treatment (1h)		**Treatment (24h)**	
Condition	10 µM Quercetin (compartment 1)	10 ng/mL LPS (compartment 3)	10 µM Quercetin (compartment 1)	10 ng/mL LPS (compartment 3)
Untreated	×	×	×	×
LPS	×	×	×	✓
Quercetin	✓	×	✓	×
LPS + Quercetin	✓	×	✓	✓

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SUPPLEMENTARY DATA

A. Risk analysis

A risk analysis was performed on the use of chemical agents required for the intracellular flavonoid accumulation assay protocol (described in section 3.3.1.2), according to the guidelines of Ghent University. The most dangerous agents (highest E-score) and their corresponding risk scores are listed below.

- Paraformaldehyde

Risk of inhalation: R = 190	- Substantially high risk – extra measures are highly recommended - Improve working place conditions where possible, if necessary perform in depth risk analysis - Activity must be carried out with higher level of caution - Perform measurements of ambient concentration in the work place atmosphere to check exposure
Risk of contact skin, eyes, mouth: R = 691	- Very high risk – stop activity immediately! - Structure improvement must be implemented immediately based on in depth risk analysis - Re-evaluate risk before restarting activities
Danger score: E = 97	

- Ethanolamine

Risk of inhalation: R = 72	- Substantially high risk – extra measures are highly recommended - Improve working place conditions where possible, if necessary perform in depth risk analysis - Activity must be carried out with higher level of caution - Perform measurements of ambient concentration in the work place atmosphere to check exposure
Risk of contact skin, eyes, mouth: R = 262	- High risk – immediate improvement is necessary - Structural measures are needed urgently based on in depth risk analysis
Danger score: E = 27	

- Triton X-100

Risk of inhalation: R = 102	- Substantially high risk – extra measures are highly recommended - Improve working place conditions where possible, if necessary perform in depth risk analysis - Activity must be carried out with higher level of caution - Perform measurements of ambient concentration in the work place atmosphere to check exposure
Risk of contact skin, eyes, mouth: R = 41	- Low risk – attention is required - Provide measures to prevent exceptional exposure
Danger score: E = 21	

- 2-aminoethyl diphenylborinate

Risk of inhalation: R = 48	- Low risk – attention is required - Provide measures to prevent exceptional exposure
Risk of contact skin, eyes, mouth: R = 17	- Very limited risk – maybe acceptable - Supervise correct implementation of the prevention measures
Danger score: E = 9	