Dietary fatty acids increase the absorption of toxic substances and drugs by modifying different absorption pathways in the intestinal epithelium

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Abstract

Dietary fatty acids have surface active properties comparable to substances used as absorption enhancers of poorly absorbed drugs. The intestinal epithelium serves as a gatekeeping barrier for the absorption of toxic substances, nutrients and drugs. In this thesis it is hypothesized that dietary fat might compromise this barrier function of the intestinal epithelium. Different absorption pathways in the intestinal epithelium, i.e. the paracellular pathway regulated by tight junctions, and the restriction of the active transcellular pathway by the efflux transporter breast cancer resistance protein (BCRP/ABCG2) were studied. Mannitol and mitoxantrone (MXR) were used as marker substances for the respective pathway. Physiologically relevant doses for humans of the important dietary fatty acids docosahexaenoic acid (DHA) and oleic acid were used. Absorption of cadmium (Cd) and aluminium (Al), relevant in a food contaminant perspective, was investigated.

DHA caused a significantly increased apical to basolateral absorption of mannitol, Cd and Al through Caco-2 cell monolayers. Moreover, oleic acid increased absorption of mannitol and Al, but not of Cd. As mannitol is a marker for paracellular absorption the findings confirm that oleic acid and DHA increase absorption of poorly absorbed substances through the paracellular pathway in Caco-2 cell monolayers. Morphological analyses with fluorescence microscopy and transmission electron microscopy supported these findings.

Oleic acid increased absorption of MXR both in Caco-2 cell monolayers and in mice. In mice, the levels of MXR were increased in blood, intestine, kidney, brain and liver. Oleic acid also caused an up-regulation of BCRP gene expression in Caco-2 cells. These findings suggest that oleic acid decrease the function of the BCRP mediated-efflux of MXR.

Overall, the results in this thesis have important toxico-kinetic implications for many food toxicants normally restricted to be absorbed through the paracellular pathway or effluxed by BCRP. The fact that dietary fatty acids increased oral absorption of toxic substances is an important finding that ought to be considered in future risk assessment. Consequently, risk-based limits for toxic substances may be underestimated if they are established in animal studies using diets with low fat content.

Keywords: oleic acid, DHA, mannitol, cadmium, aluminium, mitoxantrone, BCRP, tight junctions, Caco-2 cells, FVB mice.

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To Pontus, Emilie, Agnes, Jakob

Do not go where the path may lead; go instead where there is no path and leave a trail.

Ralph, Waldo Emerson, 1803 - 1882

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Aspenström-Fagerlund B., Ring L., Aspenström P., Tallkvist J., Ilbäck N-G., Glynn A.W., (2007). Oleic acid and docosahexaenoic acid cause an inrease in the paracellular absorption of hydrophilic compounds in an experimental model of human absorptive enterocytes. *Toxicology* 237, 12 - 23.

II Aspenström-Fagerlund B., Sundström B., Tallkvist J., Ilbäck N-G., Glynn A.W., (2009). Fatty acids increase paracellular absorption of aluminium across Caco-2 cell monolayers. *Chemico-Biological Interactions* 181, 272-278.

III Aspenström-Fagerlund B., Tallkvist J., Ilbäck N-G., Glynn A.W., (2012). Oleic acid decreases BCRP mediated efflux of mitoxantrone in Caco-2 cell monolayers. *Food and Chemical Toxicology* (2012) 50, 3635-3645.

IV Aspenström-Fagerlund B., Tallkvist J., Ilbäck N-G., Glynn A.W. (2012) Oleic acid decreases BCRP mediated efflux of mitoxantrone in mice (in manuscript).

Abbreviations

ABC transporter	ATP-binding cassette transporter
ADI	Acceptable daily intake
AhR	Aryl hydrocarbon receptor
Al	Aluminium
ATP	Adenosine triphosphate
BCRP	Breast cancer resistance protein (ABCG2)
Caco-2 cell	Colon carcinoma-2 cell
Cd	Cadmium
CYP	Cytochrome P450 superfamily
DHA	Docosahexaenoic acid
DMEM	Dulbecco´s modified eagle´s medium
DMT1	Divalent metal transporter 1
EFSA	European food safety agency
EMA	European medicine agency
EPA	Eicosapentaenoic acid
FABP	Fatty acid binding protein
FAT	Fatty acid translocase
FATP	Fatty acid transporter protein
GI	Gastrointestinal
HBSS	Hank´s balanced salt solution
IQ	2-amino-3-methylimidazol[4,5-f]quinoline
JECFA	Joint FAO/WHO expert committee on food additives
LDH	Lactate dehydrogenase
MAG	Monoacylglycerol
MRP2	Multidrug resistance protein 2 (ABCC2)
MUFA	Mono unsaturated fatty acid
MXR	Mitoxantrone
NR	Nuclear receptors

OA	Oleic acid
PBS	Phosphate buffer saline
P-gp	Permeability glycoprotein (MDR1, ABCB1)
PhIP	2-amino-1- methyl-6-phenylimidazol[4,5-b]pyridine
PPAR	Peroxisome proliferator activated receptor
PUFA	Poly unsaturated fatty acid
RNA	Ribonucleic acid
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFA	Saturated fatty acid
SLC-transporter	Solute carrier transporter
TAG	Triacylglycerol (triglyceride)
TEER	Transepithelial electric resistance
TJ	Tight junction
TWI	Tolerable weekly intake
ZIP	Zink/iron permease
ZO-1	Zona occludence-1

1 Introduction

The intake of different food constituents, e.g. fat, carbohydrates, proteins, vitamins and some trace elements, are essential for life. However, food also contains substances that are considered not to have beneficial health effects, i.e. substances that can be toxic to the body. It is also known as the basic principle of toxicology, stated in the 16th century by Theophrastus von Hohenheim, later Paracelsus, that "the dose makes the poison" or "all things are poison, and nothing is without poison, only the dose permits something to be poisonous" (Paracelsus 1493 – 1541). With today's knowledge, these statements could for some substances be questioned, for example when no threshold of toxicity for a certain effect can be estimated, e.g. mutagenicity. To elicit pharmacological, toxicological or nutritive effects a substance has to conquer different barriers of the human body to reach the systemic circulation. Certain types of food-borne substances, toxic or not, do not easily pass the intestinal barrier consisting mainly of the intestinal epithelium. However, components in food, such as fatty acids, might increase the absorption of certain substances. The consequence of this will be that a substance considered as having a low oral toxicity might be unexpectedly toxic as the available dose reaching the systemic circulation increases, i.e."the dose makes the poison".

It is well known that certain food components may alter the absorption of various drugs in the gastrointestinal (GI) tract, i.e. food is used as a help to improve absorption. There even exist guidelines how to perform food interaction studies before registration of a drug (Guideline on the Investigation of Drug Interactions (EMA, 2012)). Moreover, the pharmaceutical industry use surface active substances as excipients in drug products to improve absorption of drugs which are normally poorly absorbed through the intestinal epithelium. Because the oral route is outstanding for treatment of patients, extensive research has been done to find a solution to the problem of poor absorption of hydrophilic substances by the oral route. Based on the knowledge from

published pharmaceutical research it has been suggested that fatty acids in lipids as well as other surface active substances present in food have the ability to enhance absorption of poorly water-soluble toxic and allergenic agents (Ilback *et al.*, 2004; Mine & Zhang, 2003; Charman *et al.*, 1997). However, the impact of fatty acids on the intestinal barrier permeability has not been investigated to any great extent. As a consequence, the impact of dietary fatty acids on absorption of toxic substances from food is currently not considered in risk assessment of toxic substances.

Toxicological evaluations of most compounds are based on studies in rodents (rats, mice and rabbits) and nonrodents (dogs, pigs) that do not have the same diet as humans, i.e. fat content in the animal diets are generally lower than in the human diet, except for dogs. The possibility that surface active fatty acids in food may influence intestinal absorption of poorly absorbed substances may have an impact of the results in toxicity testing. Results from animal studies using low-fat diets may underestimate the toxicity in comparison to the human situation with high fat diets. A prerequisite for systemic toxicity of a substance in the body is that it first has to conquer the protective barrier of the intestine.

In this thesis, it is hypothesized that fatty acids common in food (oleic acid and docosahexaenoic acid (DHA)) increase absorption of poorly absorbed and food-borne toxic substances by different pathways through the intestinal epithelium. First, we investigated the impact of fatty acids on the paracellular pathway, which normally only allows small hydrophilic substances to be absorbed between the enterocytes. Secondly, we investigated the impact of oleic acid on the efflux protein breast cancer resistance protein (BCRP) situated at the apical membrane of the enterocytes, preventing substances to be absorbed by the transcellular pathway. The results demonstrate that common fatty acids in food have an impact on different absorption pathways in the GI tract, which ought to be considered in risk assessment of chemical substances in food.

2 Background

This section starts with a description of the GI tract and the absorption pathways of substances through the GI tract. Furthermore, lipids in food, the occurrence of fatty acids in food and the effects of fatty acids on the intestinal epithelium are described. A background is also given to the model substances used in the experiments. For simplification in the following text, nutrients, drugs or toxicants are cited as substances.

2.1 The gastrointestinal tract

Many organs are working in cooperation to digest food. The continuous tube of the GI tract (from the mouth to the anus) is 7 to 10 m long. A normally working GI tract is shorter due to contraction of muscles in the intestinal wall (Gad, 2007). The intestinal epithelium of the small intestine is the main site for oral absorption of substances (figure 1). The substance or food will first pass into the oral cavity, where it is processed by the teeth, the tongue and the digestive enzymes from the salivary glands, before passing through the oesophagus via the pharynx to the stomach. The food is processed in the GI tract by mechanical and chemical processes. Mechanical digestion starts in the mouth by the teeth and further down to the stomach resulting in food that is dissolved and thoroughly mixed with digestive enzymes. About 1 litre of food can be processed in the stomach at the same time. The acidity of the stomach is around pH 3 or lower. The low pH will kill bacteria entering with the food. Digestive enzymes will split large molecules such as large carbohydrates, lipids, proteins and peptides into smaller molecules (Goodman, 2010). Wave-like contractions of the smooth muscle in the wall of the GI tract force the food onwards. This contraction results in churning of the food and reduces it to a soupy liquid called chyme. Not until the food has reached the small intestine (duodenum, jejunum and ileum) from the stomach through the pylorus, is it

possible for food toxicants to be absorbed. Digestive enzymes appear along the GI tract when food passes the salivary glands, the tongue, stomach, pancreas, gallbladder and liver (Binder, 2003). During digestion, approximately 7 litres of water, acid, buffers and enzymes are secreted by the cells within the walls of the GI tract and by accessory organs into the lumen, each day. The ileum is joined to the large intestine by the ileocecal sphincter. In the large intestine, water is absorbed and residues from digestion are concentrated before expelled as faeces.

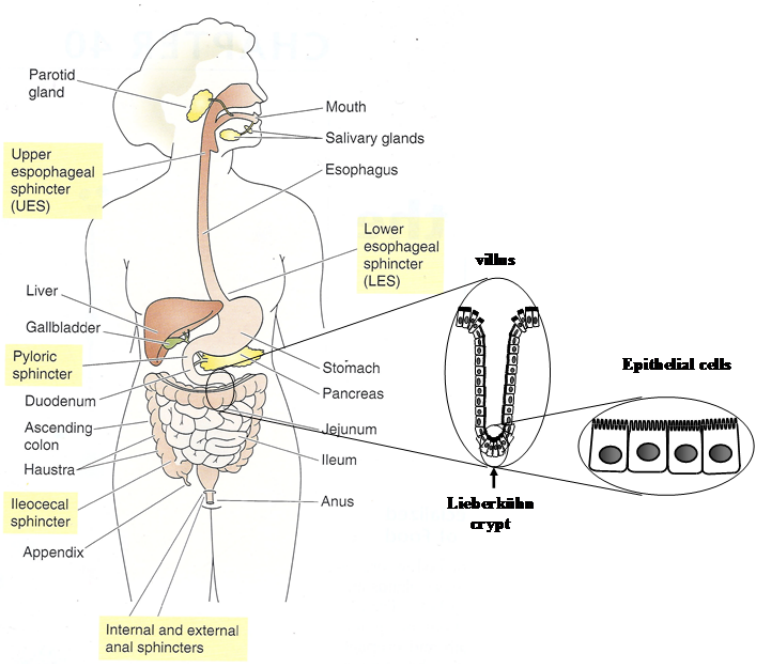

Figure 1. The major components of the human digestive system. This picture was used with the permission from the publisher. Binder H.J., Chapter 40, Organization of the gastrointestinal system, Figure 40-1, from the book Medical Physiology, Editors Boron W.F. and Boulpaep E.L., first edition, Copyright Elsevier, 2003. A villus and epithelial cells with microvilli, fingerlike protrusions at the apical side of the cells, are shown. (Modified by P Aspenström)

2.1.1 Structure of the small intestinal mucosa, the site of absorption

The small intestine consists of three parts. Duodenum (25 cm) starts at the pyloric sphincter of the stomach, and is followed by jejunum (ca 2.5 m) and lastly the ileum (2 to 4 m). In duodenum the acidic content entering from the stomach will be neutralized to a pH between 6 and 7. It takes 3 to 4 hours for the content to pass through the small intestine (Gad, 2007; Kararli, 1989).

The mucosal epithelium can be considered as a multi barrier, which has to be penetrated before the substances can reach the systemic circulation (figure 2). The epithelium regulates the flow of fluids and solutes between the interstitial space and the blood and can also be regarded as a gatekeeper, i.e. it controls the entry of nutrients and other substances.

The first absorptive barrier is the mucus layer, which consists of an outer and an inner layer. The inner mucus layer is also known as the unstirred water layer or the aqueous boundary layer and contains mostly water and a few per cent mucin (Wilson *et al.*, 1971). The glycocalyx, also called the fuzzy coat or brush border, is situated just above the enterocytes (figure 2). The glycocalyx is a viscous and elastic gel (Reitsma *et al.*, 2007; Kararli, 1989). The inner, rate limiting barrier, is the absorptive epithelium, which is lining the GI tract as a single layer of columnar cells.

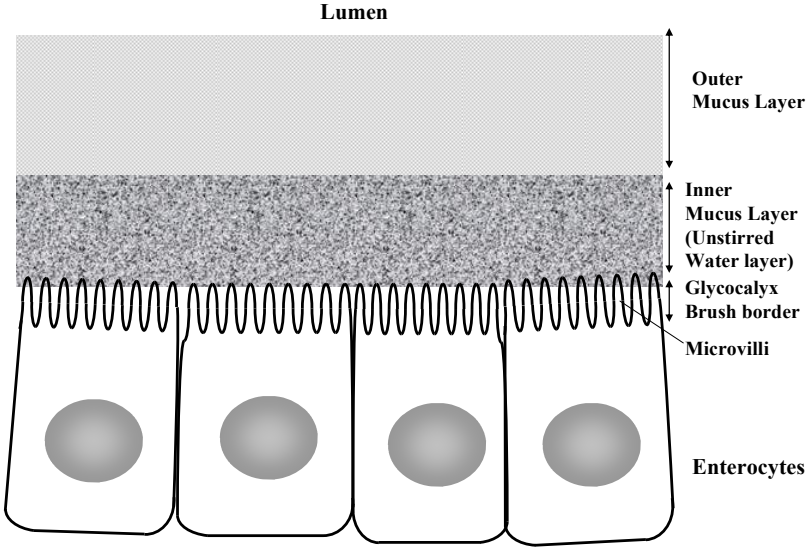

Figure 2. The intestinal barrier layers. (P Aspenström)

In order to increase the absorptive area of the intestine, the intestinal epithelium is folded into tiny finger-like protrusions, which are called villi and are 0.5 to 1 mm long (figures 1 and 2). Each villus is lined by epithelial cells, from which protrudes about 1000 microvilli on each epithelial cell, 1μm in length and 0.1 μm in diameter, this is called the brush border. Digestive enzymes are produced by the absorptive epithelial cells, and are called brush-

border enzymes which are then inserted in the plasma membrane of the microvilli (Gad, 2007; Kararli, 1989). Due to the villi and microvilli, the absorptive area of the human intestine is very large, reaching the size of a tennis court of 250 m^2 (Gad, 2007; Le Ferrec *et al.*, 2001; Kararli, 1989).

The enterocytes of the GI tract are renewed every 5 to 7 days. The enterocytes are produced through mitotic division at the basis of the villus in the crypt of Lieberkühn, from which the maturing absorptive enterocytes continuously migrate up to the tip of the villus. After approximately 3 days the enterocytes are discarded into the bulk of the intestine. The tip and the one third upper part of the villus is believed to be the main site for absorption of substances with high permeability (Gad, 2007; Kararli, 1989; Madara, 1989). When the substances are absorbed they reach the blood and then are further distributed to various sites (target organs) in the body.

The intestinal epithelium consists not only of the absorptive enetrocytes. There are three types of secretory cells called entero-endocrine cells (secrete hormones and neuropeptides), Paneth cells (located adjacent to the stem cells in the crypt of Lieberkühn, and containing antimicrobial compounds important for immunity and probably defending epithelial cell renewal) and goblet cells (secrete mucus) (Binder, 2003). In addition, there are M cells (transport intestinal bacteria and antigens from the lumen to the lymphoid tissues), caveolated cells (tuft cells) and cup cells, as well as stem cells (in crypts of Lieberkühn) and intraepithelial lymphocytes (Santos & Perdue, 2000). However, enterocytes make up to 90 % of the cells in the small intestine (Shah *et al.*, 2006; Madara, 1989).

Adjacent enterocytes are closely attached to each other by junctional complexes in the apical membrane, such as tight junctions (TJs,) (zona occludens, ZO-1), adherens junctions (zonula adherens or the intermediate junction) and the desmosomes (macula adherens) (Vandenbroucke *et al.*, 2008; Ballard *et al.*, 1995). These structures define the paracellular space which regulates the absorption of substances between the cells trough the paracellular pathway (figure 3).

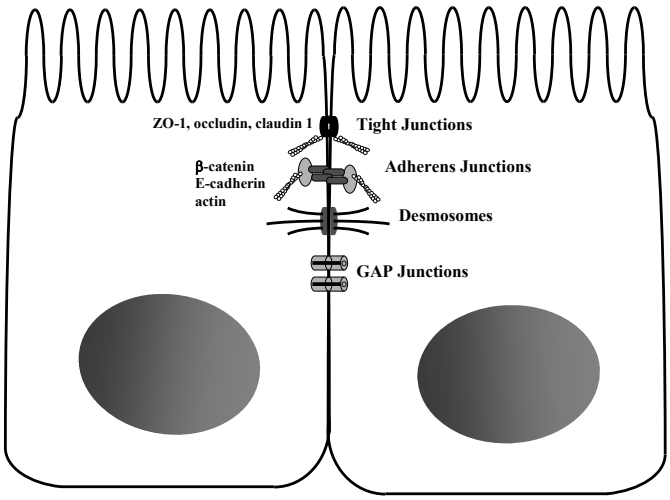

Figure 3. Different cell-cell contacts, tight junctions, adherens junctions, desmosomes and GAP junctions, between two enterocytes. Some proteins in TJs (ZO-1, Occludin, Claudin1) and in adherens junctions (β-catenin, E-cadherin) and actin are also shown. (P Aspenström)

The TJs are composed of multiple proteins, e.g. occludin, claudin 1, E-cadherin, ZO-1, caterins, actin and cingulin. Catenin is a protein involved in the formation of adherens junctions of the epithelium. Furthermore, β-catenin is necessary for the function of cadherin and its adhesive properties when binding to the cytoskeleton. Cadherins constitute a large family of single-pass transmembrane proteins principally involved in Ca^{2+} dependent cell adhesion (Gooding *et al.*, 2004).

2.2 Absorption of substances through the intestinal epithelium

When a substance has reached the small intestine, it first has to penetrate the mucus layer (Hayashi & Tomita, 2007; Larhed *et al.*, 1997). The plasma membrane below the mucus layer consists of a phospholipid bilayer, but it is also laterally segregated into different domains, consisting of a dynamic accumulation of proteins and lipids of different types. Among these are small (10 – 200 nm) sphingolipid- and cholesterol-enriched insoluble lipid rafts or membrane microdomains, important for cell signalling and intracellular lipid and protein movement (Aye *et al.*, 2009; Hayashi & Tomita, 2007; Storch *et*

al., 2007; Simons & Ikonen, 1997). The lipid rafts are tightly packed, which results in reduced fluidity as compared to the fluidity of the surrounding membrane. TJ permeability might be attained by e.g. altering the lipid composition of the cellular membrane or TJ-associated lipid rafts (Deli, 2009).

Intestinal absorption is the passage of substances through the intestinal epithelium to the circulatory system. There are different pathways for a substance to be absorbed through the intestinal epithelium (Goole *et al.*, 2010; Artursson *et al.*, 2001). The characteristics of each substance determine the way it is absorbed, i.e. its size, charge or solubility, the structure of the substance, as well as if it is hydrophilic or lipophilic. Absorption can be either passive or active. Passive transport is driven by a concentration gradient and active transport requires energy (figure 4). Energy is obtained by hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP), resulting in energy release from the phosphate bond (Goole *et al.*, 2010; Batrakova *et al.*, 2004). Three different transport processes contribute to the absorption of substances (figure 4). Transcytosis is a vesicle-mediated pathway across the enterocytes (Vandenbroucke *et al.*, 2008) (figure 4D). It is mostly large molecules such as proteins and peptide antigens that are absorbed through this pathway and they are mostly broken down by enzymes in the vesicles during the passage. The other two pathways are the paracellular and transcellular pathways (figure 4 A, B and C).

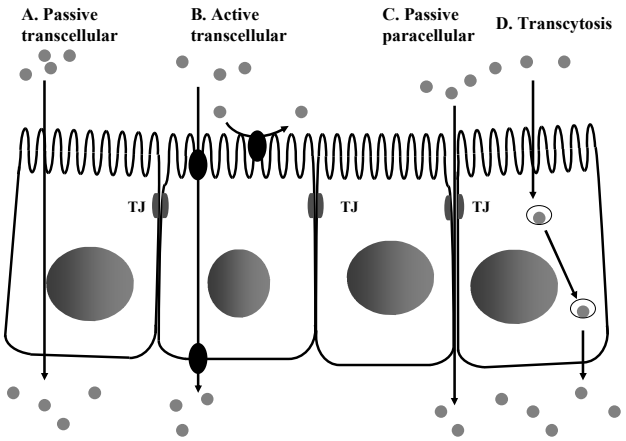

Figure 4. A substance can be absorbed through the enterocytes by different pathways. A) passive transcellular, B) active transcellular, C) passive paracellular and D) transcytosis. (P Aspenström)

2.2.1 Passive absorption through the paracellular pathway

Cell membranes are lipophilic which means that they are nearly impermeable to hydrophilic substances. The absorption of small hydrophilic substances therefore often takes place through the paracellular pathway (figure 3 and 4C). As described above, intestinal epithelial cells are closely attached to each other at the apical side of the enterocyte with TJs (figure 3). TJs surround each cell of the intestinal epithelium (Watson *et al.*, 2001). Only small or medium molecular weight, hydrophilic molecules, is absorbed through this pathway by passive diffusion (Vandenbroucke *et al.*, 2008; Artursson *et al.*, 2001). Absorption is quite limited, since the paracellular pathway comprises a very low percentage of the total epithelial surface area (Artursson *et al.*, 2001). To study TJ permeability *in vitro* the cell line Caco-2 is commonly used. Caco-2 cells are further described in the section Material and Methods.

TJs contain pores with different sizes and different charge specificities (Linnankoski *et al.*, 2010; Van Itallie *et al.*, 2008; Watson *et al.*, 2005). TJs appear to be cation selective as these ions of weak bases permeated the aqueous pores at a faster rate than anions of weak acids (Pade & Stavchansky, 1997) The pores can multiply in both small and large pores or grow larger in size, i.e. increase the pore radius. The pore radius of the very small pores is of the size of 4 Ångström (Å). Two pore sizes (5-6 Å and >10Å) have been found in the human intestinal epithelium and in Caco-2 cells (Linnankoski *et al.*, 2010). However, the paracellular porosity of the intact human intestinal epithelium is 10 times higher than in the Caco-2 cells. The number of pores was found to be much higher in the human intestine than in the Caco-2 cell monolayers, which could partly explain a lower permeability in these cells compared to the human intestinal epithelium. Watson et al., 2001, investigated the permeability of 24 polyethylene glycols (PEG) of increasing molecular radius (3.5 – 7.4 Å) in Caco-2 cells (Watson *et al.*, 2001). They found both a restrictive pore (4.3 – 4.5 Å) and a non-restrictive pore, which was responsible for permeability of larger molecules. Sodium caprate (C10) which is a fatty acid with 10 carbons in the carbon chain, had no effect on pore radius but increased permeability in another way, probably by increasing the number of functional pores. Ethylene glycol tetra-acetic acid (EGTA) treatment resulted in that cells lost all size discrimination due to increased pore size (Watson *et al.*, 2001).

TJ proteins at the apical cellular membrane, such as occludin and ZO-1, are dependent on the presence of Ca^{2+} for their function (Vandenbroucke *et al.*, 2008; Collares-Buzato *et al.*, 1994). TJs are opened through contraction of actin and myosin filaments and endocytosis of the transmembrane protein cadherin, a process that also requires Ca^{2+} (Hayashi & Tomita, 2007).

The TJ barrier is dynamic and can be modulated by both intracellular and extracellular events. Substances in food might alter or disrupt the tight junctions and in that way increase the absorption of toxic substances in food through the paracellular pathway.

2.2.2 Transcellular absorption across the intestinal epithelium

The transcellular pathway from the intestinal lumen to the circulatory system starts with the absorption of a substance through the lipid bilayer of the apical membrane of the enterocytes, followed by transport through the cytosol to the basolateral side of the membrane of the enterocytes and finally through the membrane into the circulatory system. The transport can be passive or active or both.

Passive transcellular pathway

Substances have to partition from the luminal fluid into the apical membrane of the epithelial cells before a concentration gradient driven passive diffusion takes place (figure 4A). As the cell membrane consists of lipids/phospholipids, it is mainly lipophilic substances that can be considered for this pathway. As the absorption area of the plasma membranes of the enterocytes are larger than the absorption area of the TJs, some of the expected absorption via TJs might take place by the passive transcellular pathway (Artursson *et al.*, 2001). However, the active carrier mediated transport is saturable and when this occurs substances may be absorbed through the passive transcellular route. So the absorption transcellularly can be partly active and partly passive (Sugano *et al.*, 2010; Shah *et al.*, 2006).

Active transcellular pathway

Membrane transporters have a great impact on the kinetics in the body of toxic substances and drugs but also of essential nutrients. The active transcellular pathway is mediated through transporter proteins, which function either as efflux transporters or as uptake transporters (figure 4B and 5) (Ni *et al.*, 2010; Rosenberg *et al.*, 2010; Hayashi & Tomita, 2007; Xia *et al.*, 2005). The location of many uptake and efflux transporters is at the apical side of the enterocytes, facing the lumen of the GI tract. They also reside on the basolateral side of the enterocyte, facing the blood (Giacomini *et al.*, 2010).

The transporter proteins belonging to the ATP binding cassette (ABC) family and the solute carrier (SLC) superfamily consists of more than four hundred membrane transporters in humans (Giacomini *et al.*, 2010; Anderle *et al.*, 2004). The uptake transporters, belonging to the SLC superfamily, do not

need energy as they transport their substrates according to the concentration gradient (Giacomini *et al.*, 2010; Oostendorp *et al.*, 2009). The SLC superfamily contains among others, the organic anion transporting polypeptides transporters (OATPs), organic cation transporters (OCTs) and also metal transporters.

Apical efflux transporters actively extrude substances from the epithelial cells to the intestinal lumen, and thereby reduce absorption into the systemic circulation, both parent substances and metabolic products.

The ABC family of membrane transporters functions as ATP-dependent active membrane transporters, translocating molecules across a cell membrane against a concentration gradient and thereby reducing their intracellular concentration. There are three main transporters with important clinical relevance, i.e. breast cancer resistance protein (BCRP; ABCG2, discovered 1998), multidrug resistance protein 2 (MRP2; ABCC2, discovered 1992), and permeability-glycoprotein (P-gp; MDR1, ABCB1, discovered 1976) (Doyle & Ross, 2003).

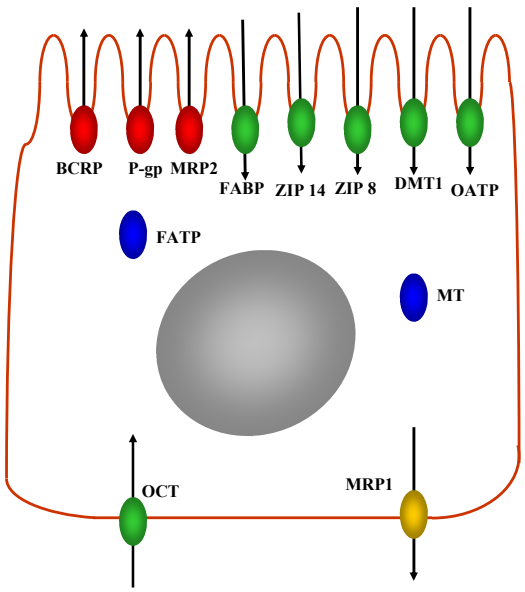

Figure 5. Examples of transporter proteins residing both at the apical side and the basolateral side of the enterocytes. Apically: the efflux transporters BCRP (ABCG2), P-gp (ABCB1), MRP2 (ABCC2), the fatty acids uptake transporter FABP, and the solute carriers DMT1, ZIP8, ZIP14 and OATP. Inside the enterocyte: the fatty acid transporter protein FATB and the metal binding protein, metallothionein MT. Basolaterally: Cd efflux transporter MRP1 and solute transporter OCT. (P Aspenström)

BCRP, P-gp and MRP2 are all present in the human intestinal epithelium at the apical side of the membrane facing the lumen (Goole *et al.*, 2010; Ni *et al.*, 2010; Rosenberg *et al.*, 2010; Aye *et al.*, 2009). Most functional ABC transporters consist of two ATP-binding domains and two sets of transmembrane domains (Doyle & Ross, 2003). ABC transporters have two sets of hydrophobic segments that cross the membrane and which are thought to assign all or most of the specificity of the transporter (Robey *et al.*, 2009).

Some transporters function as metal ion uptake transporters and belongs to the SLC family of transporters, e.g. divalent metal transporter 1 (DMT1) and the zink and iron transporter proteins (ZIP 8 and ZIP 14) (Fujishiro *et al.*, 2012). DMT1 is situated at the apical membrane and is primarily regarded as iron (Fe) transporter. However, it has been shown that it is an uptake transporter for nickel (Ni), cadmium (Cd) as well as other divalent metals (Tallkvist *et al.*, 2001).

During the last 40 years several efflux transporters have been identified of which P-gp was the first (Doyle & Ross, 2003). Three models for the efflux action of P-gp has been suggested (Constantinides & Wasan, 2007). In the pore model, substances connect with P-gp in the cytosolic compartment and are transported out through a protein channel. In the flippase model, P-gp flips drugs from the inner leaflet of the plasma membrane to the outer leaflet against a concentration gradient. Finally, in the hydrophobic vacuum cleaner model, intra membranous molecules which do not belong to the membrane, are recognised by P-gp and enter P-gp from the membranous side and leave the cell. Only sparse information of the mechanisms for the efflux action of BCRP has been found. The flip flop mechanism has been described for BCRP where the substrate was flip flopped from the outer to the inner leaflet of the cells (Breuzard *et al.*, 2007; Matsson *et al.*, 2007). It could be speculated that the same mechanisms as for P-gp are valid for BCRP as well.

P-gp has been important in the preclinical evaluation of ivermectin. It is one of the most used drugs in the world as it is important for treatment of parasitic infestations in humans and in animals. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) based the acceptable daily intake (ADI) for ivermectin on the results from a mouse strain (Mdr1a-/- mice) that had a 100-fold higher sensitivity to ivermectin than other mouse strains (Schinkel *et al.*, 1994; JECFA, 1993). This effect was shown to be due to lack of P-gp in the blood-brain barrier in this mouse strain, resulting in neurological effects and death. A lack of P-gp has also been found in certain dog species e.g. collie (sheepdogs). However, recently it has been found that ivermectin is also a substrate for BCRP (Matsson et al., 2009; de Vries et al., 2007). This

illustrates that several efflux proteins can be involved and overlapping each other for a substance.

Breast cancer resistance protein (BCRP)

BCRP is an ABC transporter and is the second member of the subfamily G, i.e. ABCG2. It has a pivotal role for absorption, distribution and excretion of drugs and potentially toxic substances that may be present in food (tables 1 and 2). During the 1990-ies a number of cell lines without overexpression of the known efflux transporters P-gp and MRP1, were found. These cell lines were resistant to mitoxantrone (MXR) (Doyle & Ross, 2003; Doyle *et al.*, 1998). One of the cell lines was derived from a resistant breast cancer cell-line and was called BCRP.

BCRP is a 75 kDa polytopic plasma membrane protein that consists of 655 amino acids. It is a half transporter with one transmembrane domain (TMD) and one nucleotide-binding domain (NBD). BCRP has to dimerize to function properly (Planas *et al.*, 2012; Giacomini *et al.*, 2010; Ni *et al.*, 2010; Wakabayashi *et al.*, 2006). BCRP has an amino acid sequence which is closely similar to one-half of the duplicated P-gp molecule. The ABCG2 gene is highly conserved and has been found in all sequenced vertebrates, including birds, reptiles, and fish.

Since the discovery of BCRP a tremendous amount of research has been done to find inhibitors and substrates for this efflux-transporter. BCRP performs energy-dependent efflux of a large number of compounds that are not structurally or chemically related (see table 1 and 2). BCRP is highly expressed in organs important for absorption (small intestine), elimination (liver and kidney), and distribution (the blood-brain and placental barriers) of drugs and xenobiotics and thereby influences the kinetics of substances and protects the body from unwanted substances (Matsson *et al.*, 2007; Gutmann *et al.*, 2005). It may also provide xenobiotic protection in stem cells (Robey *et al.*, 2009; Staud & Pavek, 2005). One physiological role of BCRP is likely to provide tissue protection against endogenous compounds as dietary flavonoids, heme, porphyrins, riboflavin, and estrogens.

Polymorphism in the BCRP gene results in inter-individual variations in the pharmacokinetic response, as well as in toxicity, caused by drugs. Inter-individual differences in BCRP function probably contribute to variable pharmacological responses of drugs that are BCRP substrates (Giacomini *et al.*, 2010). As some substances are inhibitors and some are substrates for BCRP it is possible that simultaneous exposure of BCRP inhibitors and substrates will alter the fate and toxicity of numerous drugs, carcinogens and toxicants present in food.

Surprisingly, BCRP is also situated in the apical membranes of mammary epithelial cells of the breast during lactation where it efflux substances into the milk in both humans and cows (van Herwaarden & Schinkel, 2006; Jonker *et al.*, 2005; Maliepaard *et al.*, 2001). Consequently, the suckling infant and milk drinkers may be exposed to toxic BCRP substrates through milk. A reason for this function may be to supply the infant with substances needed for growth and development like the BCRP substrates vitamin B2 (riboflavin), folic acid, vitamin K and possibly biotin (Robey *et al.*, 2009; van Herwaarden *et al.*, 2006). It could also be speculated that BCRP has a detoxifying function for the mother on expense of the health of the newborn, i.e. the survival of the mother is most important in an evolutionary perspective.

The expression of BCRP in the intestine of humans is highest in the duodenum, and then descending through the jejunum and ileum to colon towards the rectum (Gutmann *et al.*, 2005). In mice, the expression of BCRP do not follow the same pattern, i.e. it peaks in the ileum, followed by the jejunum and duodenum and then decreasing towards the rectum (Enokizono *et al.*, 2007b; Han & Sugiyama, 2006; Tanaka *et al.*, 2005).

BCRP is important in the protection of internal organs from a wide range of toxic substances that can be present in food, such as dietary carcinogens like heterocyclic amines formed during frying e.g. PhIP (2-amino-1- methyl-6-phenylimidazol[4,5-b]pyridine) and mycotoxins (ochratoxin and aflatoxin B1) (tables 1 and 2). BCRP has alo been shown to efflux trans-resveratrol 3-glucuronide which is a polyphenol from plants believed to have beneficial effects on certain diseases in humans (Planas *et al.*, 2012). Moreover, it is also an efflux transporter for many drugs used in human medicine (mitoxanthrone, estrone-3-sulfate (E_1S), nitrofurantoin, cimetidine) and in veterinary medicine (e.g. fluoroquinolones as enrofloxacin, ciprofloxacin, ivermectins).

Nuclear receptors (NR) regulate the expression of several ABC transporters (Chawla *et al.*, 2001; Schinkel *et al.*, 1994). NRs are transcription factors that function as modulators of tissue gene expression. There are forty nine members of the NR superfamily known. The mRNA expression of BCRP was directly and specifically regulated by the peroxisome proliferator activated receptors γ (PPARγ) in monocyte-derived human dendritic cells (Vlaming *et al.*, 2009). The increased gene expression of BCRP results in higher levels of the BCRP protein, which subsequently increases the capacity of cells to extrude toxic substances. This is a mechanism for cancer cells acquiring resistance against e.g. anticancer drugs (cytostatica). Seven transporters, BCRP, Abcd3 and five SLC transporters, expressed in the mouse intestine, were found to be up-regulated by PPARα (Hirai *et al.*, 2007). Thus, it seems reasonable to assume that BCRP is up-regulated by both the PPARα and PPARγ nuclear receptors.

Furthermore, expression of BCRP has also been shown to be dependent of the transcription factor, aryl hydrocarbon receptor (AhR). Known AhR agonists like 2,3,7,8-tetrachlorodibenzo-p-dioxin and benzo[a]pyrene (BP), indolo[3,2-b]carbazole increased both mRNA and protein levels of BCRP in Caco-2 cells (Tan *et al.*, 2010; Ebert *et al.*, 2005).

Table 1. Some inhibitors of BCRP (not necessarily restricted to BCRP)

Substance	References
Present in food	
Chrysin	(Mao & Unadkat, 2005; Zhang *et al.*, 2004b)
Curcumin (polyphenol)	(Kusuhara *et al.*, 2012; Zhu *et al.*, 2010);
Fumitremorgin (FTC)	(Matsson *et al.*, 2009; Allen *et al.*, 2002)
Drugs	
Geftinib (human drug)	(Zaher *et al.*, 2006)
Triclabendazole (veterinary drug)	(Barrera *et al.*, 2012)
Special synthetic inhibitors	
Ko143 (analogue to FTC)	(Matsson *et al.*, 2009; Xia *et al.*, 2005; Allen *et al.*, 2002)
Elacridar (GF 120918)	(Durmus *et al.*, 2012)

Table 2. Some substrates for BCRP relevant from a food safety point of view and some human drugs which are substrates for BCRP are shown below. Substances may be both inhibitors and substrates and some may also be substrates or inhibitors for other transporter proteins.

Substance	
Present in food	
Substances in coffee (depending on roasting time)	(Isshiki *et al.*, 2011)
Benzo[a]pyrene	(Hessel & Lampen, 2010; Ebert *et al.*, 2007; Ebert *et al.*, 2005; van Herwaarden *et al.*, 2003)
BisphenolA	(Mazur *et al.*, 2012; Ebert *et al.*, 2005)
PhIP (2-amino-1-1methyl-6-phenylimidazol[4,5-b]pyridine)	(Pavek *et al.*, 2005; van Herwaarden *et al.*, 2003)
Mycotoxins (ochratoxin, aflatoxin B1)	(van Herwaarden *et al.*, 2006)
Phytoestrogens	(Zhu *et al.*, 2010; Enokizono *et al.*, 2007a; Zhang *et al.*, 2004a)
Flavonoids (e.g. genistein sulphate, chrysin)	(Pick *et al.*, 2011; Kawase *et al.*, 2009; Zhang *et al.*, 2005; Zhang *et al.*, 2004a)
trans-Resveratrol 3-glucuronide	(Planas *et al.*, 2012)
Veterinary drugs	
Benzimidazoles (e.g. albendazole, albendazole-sulfoxide, oxfendazole)	(Haslam *et al.*, 2011; Alvarez *et al.*, 2008; Merino *et al.*, 2006; Merino *et al.*, 2005a)
Ivermectin	(Jani *et al.*, 2011; Real *et al.*, 2011; Merino *et al.*, 2009; de Vries *et al.*, 2007)
Fluoroquinolones (enrofloxacin, ciprofloxacin)	(Real *et al.*, 2011; Matsson *et al.*, 2009; Alvarez *et al.*, 2008; Merino *et al.*, 2006; Pulido *et al.*, 2006)
Human drugs and metabolites	
Methotextrate (MXT)	(Xia *et al.*, 2005)
Mitoxantrone (MXR)	(Aspenstrom-Fagerlund *et al.*, 2012; Gram *et al.*, 2009; Matsson *et al.*, 2009; Yamagata *et al.*, 2009; Yamagata *et al.*, 2007a; Zhang *et al.*, 2005; Zhou *et al.*, 2005)
Topotecan	(de Vries *et al.*, 2007; Kruijtzer *et al.*, 2002; Jonker *et al.*, 2000)
Diclofenac	(Lagas *et al.*, 2009)
Cimetidine	(Pavek *et al.*, 2005)
Estrone-3-sulfate (E_1S)	(Gram *et al.*, 2009)
17β-estradiol-glucuronide	(Mao & Unadkat, 2005)
Nitrofurantoin	(Kawase *et al.*, 2009; Merino *et al.*, 2005b)
Endogenous and essential substances	
Riboflavin	(van Herwaarden *et al.*, 2007)
Folic acid	(Assaraf, 2006; Breedveld *et al.*, 2005)
Progesterone	(Matsson *et al.*, 2009; Vore & Leggas, 2008)

2.3 Lipids in food

Michel Eugène Chevreul (1786 – 1889) was the first to discover the structure and properties of lipids. He showed that fat is generally a combination of fatty acids and glycerol forming triglycerides, and he described the structure of oleic acid, butyric acid, capric acid, stearic acid, cholesterol and glycerol. In addition, he found that lard contained solid fat which he called stearine. A liquid phase of the fat was called elaine, which was shown to be an isomer of oleine (oleic acid). All this was published in 1823, in "Recherches chimiques sur les corps gras d'origine animale" (Costa, 1962).

Dietary fat is essential for all living organisms, including humans, as a main nutrient for growth and development. It provides energy, function as a store and reservoir for lipoprotein trafficking, is important for bile acid synthesis, stereoidogenesis and is also a structural component in cells, e.g. in the cell membrane (Iqbal & Hussain, 2009). Fatty acids also regulate gene expression for nuclear receptors and transporters.

In a balanced diet about 30 to 35 % of the energy (E %) consist of fat and more than 90 % of the dietary fat comprises triacylglycerol (TAG) or triglycerides (EFSA Panel on Dietetic Products, 2010). In Sweden the intake of fat is 34.2 E %, based on Riksmaten 2012 (National Food Agency, 2012). Phospholipids, sterols (e.g. cholesterol), and other lipids (e.g. fat soluble vitamins) are the remaining constituent in dietary fat (Ratnayake & Galli, 2009). TAG consists of three fatty acids connected to a glycerol molecule with ester bonds (figure 6). The most common fatty acids in food are long chain fatty acids (>12 carbons) (Goodman, 2010).

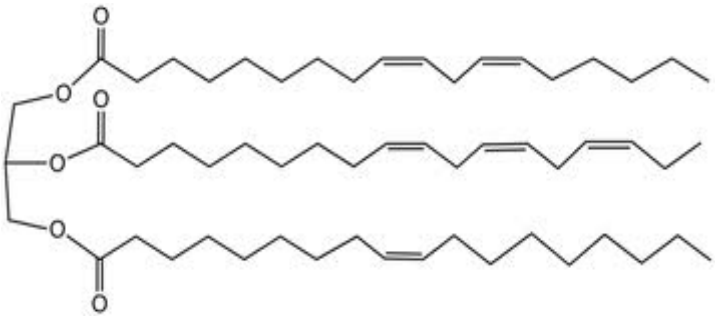

Figure 6. Triglyceride with linoleic acid (C18:2) at the top, linolenic acid (C18:3), in the middle and oleic acid (C18:1) at the bottom.

2.3.1 Fatty acids

A fatty acid consists of a carbon chain starting with a carboxyl group at one end and a methyl group in the other end. Fatty acids are divided in groups of

different chain lengths. The short-chained fatty acids have 3 to 7 carbons, the medium-chained fatty acids have 8 to 13 carbons, and the long-chained fatty acids have 14 or more carbons (Ratnayake & Galli, 2009). Saturated fatty acids (SFA) are devoid of any double bond between the carbons in the carbon chain. There are also monounsaturated fatty acids (MUFA) with one double bond and polyunsaturated fatty acids (PUFA) with several double bonds. When the first double bond is situated at the third, sixth or ninth carbon from the methyl group, the fatty acid belongs to the Ω-3, Ω-6 or Ω-9 fatty acids (Ratnayake & Galli, 2009). The melting point of fatty acids decreases with increasing number of double bonds and the reactivity increases with increasing number of double bonds. The body lacks enzymes for desaturation of double bonds at position 3 and 6, which means that these fatty acids are needed from the diet, i.e. essential fatty acids. The parent fatty acid from the Ω-3 family is α–linolenic acid [18:3] and from the Ω-6 family it is linoleic acid [18:2]. However, the body is able to endogenously produce SFA and indirectly Ω-9 fatty acids, by synthesis from carbohydrates (Ratnayake & Galli, 2009).

In addition to the involvement in cell structure and energy supply, dietary fat has a great impact on gene expression, regulating metabolism, growth and cell differentiation (Jump, 2004; Chawla *et al.*, 2001; Jump & Clarke, 1999; Jump *et al.*, 1997). Several members of the NR super family have been found to be fatty acid receptors, among others the PPARs (Khan & Vanden Heuvel, 2003; Jump & Clarke, 1999). Several mono- and polyunsaturated fatty acids bind to PPARα at physiological concentrations and cause transcriptional activation (Hirai *et al.*, 2007; Khan & Vanden Heuvel, 2003; Chawla *et al.*, 2001).

Metabolism of lipids in the GI tract

Triglycerides are digested in the intestine to mono-, di- and triglycerides and free fatty acids (FFA). The cooking of food, chewing of food, and churning and peristalsis in the stomach facilitate the formation of an emulsion of the triglycerides. Emulsification of dietary fat also involves lingual and gastric enzymes, but only approximately 15% of the fat is digested in the stomach (Iqbal & Hussain, 2009). Crude emulsion of fine lipid droplets mainly containing triglycerides, reach the duodenum, where they are further hydrolysed by pancreatic lipases. The hydrolysis, mediated by pancreatic lipases, starts with the first and third fatty acid chain at the glycerol molecule, leaving two free fatty acids and one 2-monoacylglyceride (2-MAG) (Goodman, 2010). During the hydrolysis, emulsion droplets dissociate into micelles with the help of bile salts (Goodman, 2010). These micelles are carried across the unstirred water layer above the brush border membranes of

the enterocytes. The mixed micelles then reach the lipid bilayer in the apical membrane of the enterocytes, where the fatty acids are either protonated and leave the mixed micelle to diffuse across lipid bilayer membranes, or become provisionally a cell membrane lipid.

Different mechanisms have been suggested for the uptake and transport of long-chained fatty acids across the apical membrane of the enterocytes. One is the protein-independent diffusion model and another is a protein-dependent mechanism (Iqbal & Hussain, 2009). One important protein for the uptake of fatty acids in the enterocyte is the fatty acid translocase (FAT or FAT/CD36), which is expressed in the intestinal epithelium. The presence of dietary fat as well as genetic obesity and diabetes, results in an up-regulation of FAT. Furthermore, the fatty acid transporters FATP2 (SLCA272) and FATP4 (SLCA274) have been found to be expressed in the small intestine (Goodman, 2010; Iqbal & Hussain, 2009; Mu & Hoy, 2004). The fatty acid-binding protein family (FABP) is tissue specific and is situated in many tissues, and also in the brush border membrane of the enterocytes. The FABPs has high affinity for binding of long-chain fatty acids and may play a role in the uptake of fatty acids (Storch & Thumser, 2010). Once inside the enterocytes long chained fatty acids and MAG are carried by the fatty acid transport protein (FATP) and cross the cytoplasm to the smooth endoplasmatic reticulum for reconstitution to form triglycerides (TAG). Several enzymes take part in this synthesis to TAG (Iqbal & Hussain, 2009; Mansbach & Gorelick, 2007). After the fatty acids have been re-synthesized to TAG in the enterocyte they are assembled with proteins and phospholipids into chylomicrons. Several transport proteins are involved in this process. Finally, the chylomicrons are expelled across the basolateral membrane to the lymph vessels in the core of the villus by exocytosis and the TAG enters the bloodstream via the thoracic duct, the largest lymphatic vessel in the body.

Surface activity of fatty acids

Fatty acids have different characteristics depending on carbon length and number of double bonds. The number of carbons, as well as the number of double bonds, is important for the surface activity of fatty acids (Mao & Unadkat, 2005). Fatty acids, like other surface active substances, have a lipophilic part and a hydrophilic part. The hydrophobicity of fatty acids increases with carbon chain length. This is consistent with Traube's rule which states: "in dilute aqueous solutions of surfactants belonging to any one homologous series, the molar concentrations required to produce equal lowering of the surface tension of water decreases threefold for each additional CH_2 group in the hydrocarbon chain of the solute" (Attwood, 2006).

2.3.2 Intake of fatty acids through the diet

Saturated fat on average represents about 13 E % of the Swedish diet or 40 E % of the total fat, as a component in hard margarines, butter, meat and dairy products (Livsmedelsverket (2012); Becker & Pearson, 2002). Palmitic acid [C16:0] and stearic acid [C18:0] is quantitatively the most important of the saturated fatty acids (SFA).

Monounsaturated fatty acids (MUFA) represents approximately 12.8 E % of the total diet and 39 E % of the total fat in food (Livsmedelsverket (2012)). The quantitatively most important MUFA is oleic acid [C18:1], which can be found in milk fat and vegetable oils up to 71% (EFSA Panel on Dietetic Products, 2010).

Polyunsaturated fatty acids (PUFA) represents approximately 5.6 E % of the total diet or 14 E % of the total fat in the diet (Livsmedelsverket (2012)). The PUFA's are essential fatty acids that are present in vegetable oils, soft margarines and fish oils. Ω-3 fatty acids is found in linseeds, rapeseed oil and walnuts, fish, human milk and marine algae (EFSA Panel on Dietetic Products, 2010). The most commonly discussed Ω-3 fatty acids are docosahexaenoic acid (DHA), with an average intake of 0.4 g/day, and eicosapentaenoic acid (EPA), with an average intake of 0.2 g/day.

2.3.3 Fatty acids used in the thesis

In our studies we have used oleic acid and DHA. Oleic acid was used because it is the most common MUFA in food, whereas DHA is interesting in a health perspective, i.e. it is sold as Ω-3 food supplement that contains high amounts of DHA and EPA.

Oleic acid

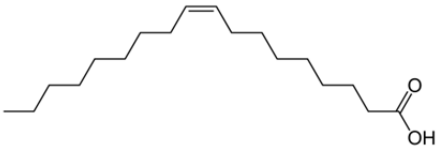

Figure 7. Oleic acid (C18:1, n=9) Mw 282.5

Oleic acid is the most common fatty acid in food, in Sweden, with a contribution of approximately one third of the total intake of fat (Becker & Pearson, 2002). It is present in both plant and animal derived foods (including fish), in seed, avocado, nuts, milk fat (21 mg/100g), olive oil (71.3 mg/100g),

and a lot of other vegetable oils (Rose & Connolly, 1999). Oleic acid can be synthesised by mammals.

Oleic acid has been associated with a positive effect on stroke and coronary heart disease (Samieri *et al.*, 2011; Waterman & Lockwood, 2007). It has also been shown that olive oil induces its beneficial effect on blood pressure through oleic acid (Teres *et al.*, 2008). Olive oil is also believed to reduce the incidence of cancers of the breast, skin and colon (Owen *et al.*, 2004).

The typically daily intake in Sweden of MUFA (oleic acid) has increased during 2005 to 2010 from approximately 39.1 to 42.1 g/person daily based on data from a market food basket (Livsmedelsverket (2012)). Based on data from a population-based food consumption survey, Riksmaten 2010 -2011, an average daily intake of 24.2 g (woman) to 30.2 g (male) of oleic acid was estimated, corresponding to 85.6 - 107 mmol a day or 28.5 – 35.6 mmol/meal (National Food Agency, 2012). The actual oleic acid level in the intestine depends on the volume of the stomach content at the time of intake. A volume of 0.1 litres to 1 litre in the stomach after a meal would on average result in oleic acid levels ranging from 285 - 356 mM to 28.5 – 35.6 mM. As described above, pancreatic lipase in the duodenum hydrolyses fatty acids at position 1 and 3 in the TAG, leaving 2 fatty acids and one monoglyceride (Goodman, 2010). Consequently, after ingestion of for instance 28.5 mM oleic acid bound in triglycerides, approximately 19 mM will be hydrolysed to free fatty acids in the GI tract.

Docosahexaenoic acid (DHA)

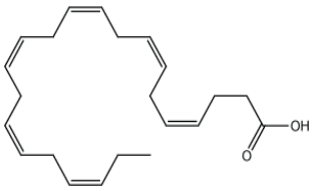

Figure 8. DHA (C22:6, n=3), Mw 328.5

DHA belongs to the Ω-3 fatty acids. It is found in fish, shellfish, marine algae, marine mammals, human milk, egg and liver. It cannot be synthesised by mammals and must therefore be solely obtained from the diet, which means that it is an essential fatty acid (Rose & Connolly, 1999). However, new knowledge challenges this suggesting that DHA and EPA can be synthesized from the essential fatty acid α-linolenic acid (C18:3) (Ratnayake & Galli, 2009). DHA and EPA are involved in many physiological processes, e.g.

modulation of inflammation, platelet aggregation, immune responses, cell growth and proliferation, and contraction and dilation of smooth muscle cells (Ratnayake & Galli, 2009). A protective role of Ω-3 fatty acids in cancer development has also been discussed (Rose & Connolly, 1999).

Adverse effects of high intakes of DHA and EPA have been reported as bleeding episodes, impaired immune function, impaired lipid and glucose metabolism and increased lipid oxidation (EFSA Panel on Dietetic Products, 2012). Based on benefits to reduce cardiovascular disease risk, the EFSA panel recommended daily doses between 0.25 to 0.5 g of EPA and DHA. However, in contrast to this the panel is also of the opinion that supplemental intakes of a combination of DHA and EPA up to 5g daily would not cause safety-related problems for the adult population. In addition, supplemental intakes of 1 g DHA alone/day were estimated to not give safety concerns in the general population.

The typically daily intake in Sweden of PUFA has increased between 2005 and 2010 from approximately 14.2 to 15.3 g/person and day based on data from a market food basket (Livsmedelsverket (2012)). The Ω-3 fatty acids comprised 21.6% of the total PUFA intake, or 3.3 g/person a day. The intake of α-linoleic acid, which is the parent fatty acid of the long-chained Ω-3 fatty acids in the body, is presently 2.6 g/person/day (National Food Agency, 2012). The mean intake of DHA between 1997 and 1998 was 0.21 to 0.24 g/day for females and males, respectively (Becker & Pearson, 2002). Consumption of a meal of 100 g farmed rainbow trout resulted in an intake of 3 mmol DHA (1 g/100 g) and 1.7 mmol (0.5 g/100g) EPA (Becker & Pearson, 2002). A volume of 0.1 litre to 1 litre in the stomach after a meal would on average result in a DHA level of 3 to 30 mM. As a comparison, consumption of 100 g rainbow trout corresponds to four capsules of some Omega-3 supplements, half the daily dose which was recommended by the manufacturer, i.e. 6 mmol DHA/EPA.

2.3.4 Influence of fat, fatty acids and surfactants on absorption of substances

Fat-rich meals have been shown to increase absorption of certain drugs used in human and veterinary medicine. Several anthelmintic substances are poorly absorbed by the oral route, which is an advantage for the treatment, as the parasites exist in the GI tract lumen. When ivermectin was taken together with a fatty meal (48.6 g), a 2.5-fold increased bioavailability was shown in humans (Guzzo *et al.*, 2002). Increased bioavailability of triclabendazole was observed when patients received a high energy breakfast (Lecaillon *et al.*, 1998). The bioavailability of albendazole increased 4.25-fold after a fatty meal, compared to when given in a fasted state (Lange *et al.*, 1988). Furthermore, the effect of a

"local fatty breakfast" on the bioavailability of albendazole was examined in a cross over design, which showed that the bioavailability of albendazole sulphoxide was increased four-fold when it was given with a fatty meal compared to the fasting state (Awadzi *et al.*, 1994).

Ivermectin is a known inhibitor of P-gp and recently it was shown to be an inhibitor of BCRP as well (de Vries *et al.*, 2007; Matsson *et al.*, 2007). The benzimidazoles, triclabendazole and triclabendazole sulfoxide, are substrates for P-gp, whereas albendazole and albendazole sulfoxide is substrates for BCRP (Dupuy *et al.*, 2010; Merino *et al.*, 2009; Merino *et al.*, 2005a).

Several studies have shown that surfactants interact with the function of efflux proteins and thereby increase absorption of certain substrates for BCRP (Hirunpanich & Sato, 2009; Wempe *et al.*, 2009; Zhang *et al.*, 2008; Constantinides & Wasan, 2007; Yamagata *et al.*, 2007b; Bogman *et al.*, 2005). As mentioned before attempts have been made in pharmaceutical research and academia to increase oral bioavailability of poorly absorbed drugs (Oostendorp *et al.*, 2009; Robey *et al.*, 2009; Anderberg *et al.*, 1993). Medium-chain fatty acids (C8 to C12) have induced a dose-dependent increase of absorption of the paracellular marker mannitol across Caco-2 cell monolayers (Chao *et al.*, 1999; Lindmark *et al.*, 1998a). It was also found that sodium caprate (C10) induced leakier TJs as measured by trans-epithelial electrical resistance (TEER) (Lindmark *et al.*, 1998b). Moreover, C12-fatty acids were the most effective absorption enhancers among the medium-chained fatty acids (Cano-Cebrian *et al.*, 2005). Oleic acid (C18) was shown in Caco-2 cell monolayers to increase iron uptake and also to increase absorption of Lucifer yellow, a fluorophore which is a marker for paracellular absorption, (Droke *et al.*, 2003).

The surface active properties of fatty acids imply that they can be compared with surface active substances used as absorption enhancers in the pharmaceutical industry. In conclusion, the impact of fatty acids on BCRP efflux or other transporter protein efflux has not been investigated thouroughly.

2.4 Possible mechanisms of fatty acids on intestinal absorption

Paracellular absorption

Fatty acids may affect the integrity of TJs by several ways and thereby increase the paracellular absorption.

Calcium ions are important for the function of TJs as these are disrupted if the extracellular or intracellular concentration of Ca^{2+} is reduced (Ma *et al.*, 2000). Complex-binding of Ca^{2+} by fatty acids might reduce the amount of Ca^{2+} available for normal function of TJ. This would result in a leakier TJ that allow larger molecules to pass. Fatty acids may also bind Ca^{2+} and thereby

cause the formation of biologically inert soaps which reduce the amount of Ca^{2+} available for normal function of TJs (Anderberg *et al.*, 1993). The intracellular presence of Ca^{2+} is also important for the formation of TJs and for the opening of TJs (Hayashi & Tomita, 2007; Collares-Buzato *et al.*, 1994). In addition, some cat-ionic substances like Cd^{2+} may compete with Ca^{2+} and in that way disrupt the TJs.

Other possible mechanisms can include the importance of acidity (pH) for the integrity of the cell membrane. The pH partitioning theory means that a phospholipid bilayer only can be permeated by an uncharged molecule. However, it has later been shown that charged molecules can be absorbed through the paracellular route (Nagahara *et al.*, 2004). In *in vitro* studies it is important to control the acidity in the experimental medium as an acidity below pH 5 or above pH 8 will damage the cell membranes (Nagahara *et al.*, 2004).

Fatty acids may, like other surface active substances, penetrate the cell membrane, destabilize the membrane and alter the structure of it, resulting in an increased absorption. When the structure of the cell membrane is impaired it will disturb the cell-cell contacts and thereby influence the integrity of the TJs. This effect seems to be stronger the longer the carbon chain is and the more double bonds the fatty acid possesses, i.e. Traube's law, see 2.3.1 above.

One mechanism could be that fatty acids increase the lipophilicity of a substance and therefore it is easier for the substance to diffuse through the mucus layer and UWL and reach the enterocytes. This can be tested by octanol/water partitioning experiments, see Cd experiments below.

TJs consist of pores with different sizes. Fatty acids have been shown to increase the number of functional pores, both large and small (Watson *et al.*, 2001). It is also possible that fatty acids increase the radius of the pores, thus letting larger molecules pass and increase the absorption in that way.

Active transcellular absorption

BCRP and other efflux transporters are situated in the apical membrane of the intestinal cells and Caco-2 cells. If a substance functions as inhibitor of BCRP the intestinal absorption of a substrate for BCRP increases. Thus, the efflux mediated by BRP will be reduced. Possible mechanisms by which surfactants inhibit BCRP are not known. Nevertheless, there are some mechanisms that might be possible.

To work properly, active efflux requires energy. It could be speculated that fatty acids cause an inhibition of ATP hydrolysis, by inhibiting ATPase activity and in that way decreases efflux of the BCRP substrate. It has been shown that the surfactant Pluronic P85 inhibited, in a dose-dependent manner,

ATPase activity in suspensions of P-gp overexpressing membranes (Batrakova *et al.*, 2004).

Fatty acids may act as substrates or inhibitors of BCRP. Butyrate, a short chained (C4) fatty acids was shown to be a substrate for BCRP (Goncalves *et al.*, 2011). It could be hypothesized that simultaneous exposure of fatty acids and BCRP substrates or inhibitors will give a competitive inhibition of BCRP. It could also possible that the efflux transporter can be saturated and in that way reduce the efflux function.

Another mechanism could be that fatty acids are incorporated in the cell membrane and are able to modulate the activity of efflux transporters, by altering the membrane function around the transporter (Aye *et al.*, 2009). It has been shown that P-gp is partly localized to lipid rafts and that the cholesterol and lipid composition of the cell membrane is essential for the function o P-gp (Troost *et al.*, 2004). BCRP was also demonstrated to be mainly situated in lipid rafts in the cell membrane and that cholesterol may considerably alter the efflux activity of BCRP *in vitro* in an MDCKII (canine kidney) epithelial cell-line (Storch *et al.*, 2007).

Structure specificity is also an important factor for inhibition of transporters by surfactants. Only Tween 20 of the polysorbate family of surfactants was shown to increase MXR accumulation in MDCK-II cells (Yamagata *et al.*, 2007a).

The impact of fatty acids on the PPAR-α and –γ, which in turn up-regulates the expression of BCRP seems to be important for the function of BCRP and probably other efflux transporters (Montagner *et al.*, 2011; Hirai *et al.*, 2007).

2.5 Tested Substances

Three substances were selected for investigations of the influence of fatty acids on the absorption through the paracellular pathway, i.e. cadmium (Cd) (paper I), mannitol (papers I and II) and aluminium (Al) (paper II). MXR was chosen as a model substance in the studies of the effect of oleic acid on BCRP-mediated efflux (papers III and IV).

2.5.1 Cadmium (Cd)

Cd is a divalent cation but it is often found in complexes with other substances in food e.g. chloride (Cl^-). The main source of Cd exposure is through food, i.e. approximately 90% for non-smoking humans (Bergeron & Jumarie, 2006). In a survey of drinking water and foods in Europe, drinking water normally contained the lowest amount of Cd (0.001 μg/kg) while horse kidney contained the highest amount of Cd (61 mg/kg) (EFSA, 2012). It was found that the 5th

percentile intake was 2.1 μg Cd/kg bw weekly and the 95th percentile intake was 12.1 μg Cd/kg bw weekly (EFSA, 2012).

Long term human exposure to low doses of Cd results in an accumulation of Cd in the proximal tubules in the kidney, which might result in irreversible renal failure. In addition, an increased risk of cancer has been indicated. Cd also accumulates in bones. The half-life for Cd excretion is 10 to 30 years. The reference point for establishing a tolerable weekly intake (TWI) was 1μg Cd/g creatinine in urine. In order not to exceed this reference point the average daily dietary Cd intake should be below 0.36 μg/kg bw, which results in a TWI of 2.5 μg/kg bw, as established by EFSA in 2009 (CONTAM, 2011).

Cd might be absorbed through the GI tract either by the passive paracellular route, the passive transcellular route or the active transcellular route (Endo *et al.*, 2000; Blais *et al.*, 1999; Jumarie *et al.*, 1999). Absorption of Cd is known to be regulated by transporter proteins, e.g. the divalent metal transporter 1 (DMT1, SLC11A2) (Elisma & Jumarie, 2001; Tallkvist *et al.*, 2001), ZIP8 and ZIP14 (Fujishiro *et al.*, 2012; Vesey, 2010; Elisma & Jumarie, 2001; Tallkvist *et al.*, 2001). DMT1 is present in the apical membrane of Caco-2 cell monolayers (Elisma & Jumarie, 2001; Tallkvist *et al.*, 2001). Cd is also bound with high affinity to the intracellular metal-binding protein metallothionein (MT), and is regarded as the most potent inducer of this protein in many tissues, e.g. enterocytes, liver and kidney (Tallkvist *et al.*, 2001; Rossi *et al.*, 1996). A dose-dependent increase in P-gp expression was shown in Caco-2 cells after 4 weeks exposure to 1 and 5 μM Cd indicating that Cd influences P-gp expression (Huynh-Delerme *et al.*, 2005). Cd ions compete with Ca^{2+} ions which normally regulate the formation and sealing of TJs (Rossi *et al.*, 1996). Recently, it was shown that $CdCl_2$ inhibit the efflux function of MXR and therefore had an inhibitory effect of the function of BCRP (Kummu *et al.*, 2012). However, the picture of transporter proteins involved in Cd absorption is far greater than described here.

Cd is known to be poorly absorbed through the intestinal epithelium. It was earlier estimated that only a fraction of 3 to 7% of the Cd found in food was absorbed in humans and only 0.3 to 3.5% in adult rats (Vesey, 2010). An average absorption of 5% was taken into account when EFSA established a TWI for Cd in 2009 (EFSA, 2009). However, diet composition and nutritional status has a great impact on intestinal Cd uptake. It was reported that a high dietary concentration of lipid and protein increased the net intestinal uptake of Cd (Andersen *et al.*, 2004).

2.5.2 Mannitol

Mannitol (mw 182.17) is a sugar alcohol, derived from reduction of sugar, ($C_6H_8(OH)_6$). It is for example used in medicine as an osmotic diuretic and also for reducing intracranial pressure. It is also used as a food additive (E421) and sweetener, where the poor absorption is beneficial for its use. Mannitol is exclusively absorbed through the TJs, i.e. the paracellular pathway, and is therefore commonly used as a marker for the paracellular absorption (Hubatsch *et al.*, 2007; Anderberg *et al.*, 1993).

2.5.3 Aluminium

Aluminium (Al) is naturally present in food in low concentrations (µg/kg), in tea, cocoa, herbs, spices and drinking water (Yokel *et al.*, 2008; Jorhem & Haegglund, 1992). However, food can also be contaminated during processing, cooking and storage, thereby resulting in a rather high intake for consumers, i.e. 90 mg Al/week in Sweden (Jorhem & Haegglund, 1992). Al is also included in food additives (e.g. anticaking powder) and in drugs (e.g. vaccines, antacids), both for human and veterinary use (Crisponi *et al.*, 2012; Greger & Sutherland, 1997). A tolerable weekly intake of 1 mg Al/kg bw has been established by both EFSA (EFSA Panel on food additives, 2008) and JECFA (JECFA, 2006). The TWI was based on the no-observed-adverse-effect level (NOAELs) in mammals for neurotoxicity, embryotoxicity and testicular toxicity.

Al is generally insoluble at neutral pH but can be solubilised by complexing agents, such as citrate, lactate, fluoride and silicate present in food (Zhou & Yokel, 2005; Glynn *et al.*, 2001; Froment *et al.*, 1989; Slanina *et al.*, 1986). Al has, in some studies, been shown to be absorbed by the paracellular route (Aspenstrom-Fagerlund *et al.*, 2009; Zhou & Yokel, 2005; Whitehead *et al.*, 1997; Provan & Yokel, 1988). Recently an influx transporter, Nrat1 (a member of the Nramp family of transporters) was found to function as an Al influx transporter in rice, specified for trivalent ions (Xia *et al.*, 2011). However, no information of other transporters for aluminium was found.

Certain diseases or physical conditions might modify toxicokinetics of Al and thereby increase its absorption and bone deposition (Crisponi *et al.*, 2012). The risk assessment assumes a low absorptive bioavailability of Al, which is less than 1% in laboratory animals and humans (Yokel & McNamara, 2001; Greger & Sutherland, 1997). In the risk assessment of Al, the impact of food on the bioavailability has not been taken into account.

2.5.4 Mitoxantrone

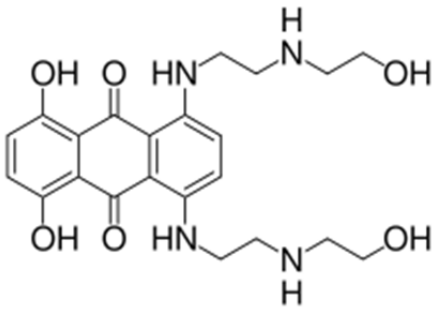

Figure 9. Mitoxantrone (MXR), 1,4-dihydroxy-5,8-bis[2-(2-hydroxyethylamino) ethyl amino]-anthracene-9,10-dione, $C_{22}H_{28}N_4O_6$, Mw 444.48

MXR is an anthracenedione, antineoplastic agent used for example in the treatment of breast cancer, acute leukemia's and non-Hodgkin's lymphoma in humans and animals since the 1980th (Batra *et al.*, 1986). MXR is genotoxic and acts as a topoisomerase II inhibitor, disrupting DNA synthesis and DNA repair in both healthy cells and cancer cells (Boos & Stopper, 2000; Stopper *et al.*, 1999). As a therapeutic agent MXR is administered by the parenteral route, since absorption by the oral route is not efficient enough (Scott & Figgitt, 2004; IARC, 2000; Batra *et al.*, 1986). The low oral bioavailability is due to the presence of BCRP in the intestine (Matsson *et al.*, 2009; Doyle & Ross, 2003). Thus, MXR is often used as a model substance to study BCRP-mediated efflux of substances (Matsson *et al.*, 2007; Yamagata *et al.*, 2007a; Yamagata *et al.*, 2007b; Cisternino *et al.*, 2004).

3 Aims of the thesis

The overall aim was to examine whether dietary fat increase absorption of food toxicants by modifying the barrier functions of the intestinal epithelium. The impact of food components, like fatty acids, on absorption of toxic substances is currently not taken into consideration in risk assessment.

The specific aims were:

1. To investigate *in vitro* if fatty acids common in food, i.e. oleic acid and DHA, compromise the integrity of the intestinal epithelium causing increased paracellular absorption of poorly absorbed hydrophilic substances such as mannitol, cadmium and aluminium (Papers I and II).

2. To investigate *in vitro* and *in vivo*, if oleic acid negatively affects the function of the efflux transporter BCRP by investigating the absorption of the BCRP substrate MXR and to investigate the influence on gene expression of BCRP (Papers III and IV).

4 Materials and methods

An overview of the methods used in this thesis is given in this section. Details and the full description of the experiments are given in the individual papers included as papers I to IV.

4.1 Experimental models

The effect of fatty acids, commonly present in food, on transport of poorly absorbed toxic substances either by the passive paracellular pathway or the active transcellular pathway was investigated both *in vitro* and *in vivo*. Caco-2 cell monolayers were used in the *in vitro* investigations and FVB male mice in the *in vivo* investigations.

4.1.1 Human intestinal epithelial enterocytes, Caco-2 cell monolayers (Papers I, II, III)

The Caco-2 cell model is an experimental model of human absorptive enterocytes. Caco-2 cells are derived from a human primary colorectal adenocarcinoma from a 72 year old patient (Fogh *et al.*, 1977). Caco-2 cells are widely used for studies of absorption of drugs, nutrients and toxic substances, both in the academia and in the pharmaceutical industry. Searching Pubmed, using the search term "Caco-2" gives more than 10 000 hits (July 2012). When fully matured, the Caco-2 cells are polarized into an epithelial cell monolayer that provides a physical and biochemical barrier to the passage of ions and molecules. The reason why cancer cells are used is because they can be grown for longer time than normal cells which only survives for approximately 10 days when cultivated.

When Caco-2 cells are seeded on permeable filters and placed in bicameral chambers, they differentiate spontaneously, polarize and form monolayers. During the differentiation phase the monolayers develops a phenotype similar

to the small intestinal absorptive enterocytes with a well-differentiated brush border (Artursson *et al.*, 2001; Pinto, 1983). The monolayers with its brush border express typical small intestinal microvilli hydrolases, enzyme systems, transporters for absorption and efflux, as well as tight junctions with typical tight junctional proteins (Englund *et al.*, 2006; Delie & Rubas, 1997; Meunier *et al.*, 1995). The correlation of gene expression of ten efflux proteins was compared between the human jejunum and Caco-2 cell monolayers (Taipalensuu *et al.*, 2001). A good correspondence between Caco-2 cells and human jejunum for 9 of 10 transporter proteins (including P-gp) was found. However, less agreement was found for BCRP which had a 100-fold lower transcript copy number in Caco-2 cells compared to human jejunum. Both the monomer and dimer of BCRP are expressed in fully differentiated Caco-2 cells and situated at the apical side of polarized Caco-2 cells (Ebert *et al.*, 2007; Yamagata *et al.*, 2007b; Xia *et al.*, 2005).

The monolayers do not produce mucus, which means that apical exposure to substances will take place without this protecting layer in Caco-2 cell experiments. Independent measures of test substances on the apical side (directed towards the intestinal lumen) and the baso-lateral side (directed towards the blood) of the monolayer can easily be carried out.

Culturing and differentiation of Caco-2 cells

The Caco-2 cells were a kind gift from Professor Artursson, Department of Pharmacy, Uppsala University. Cells were grown mainly according to the protocol for culturing of Caco-2 cells designed by that group (Hubatsch *et al.*, 2007). In the current experiments Caco-2 cells were first grown to reach confluency in plastic flasks (25 and 75 cm^2) in a cell incubator under standardized conditions. At confluence, which takes two to three days, cells were detached with trypsin and split at a 1:2 ratio into two new flasks or seeded on filters. A passage means the number of times that cells have been sub-cultured into a new flask. In our experiments a rather high passage, 92 to 97, was used. In some early experiments we used passage 50 to 100. Cells were seeded on Falcon filters in six-well plates, and with a cell density of 1.5 to 2.0 x 10^6 cells per filter. The culture medium was changed both apically and basolaterally every second or third day. The step of growing on the filters is called the differentiation phase and it takes 20 to 25 days to get fully differentiated monolayers with the characteristics of the small intestinal epithelium. If cells grow too long they start forming multilayers and are then not suitable to use in transport experiments.

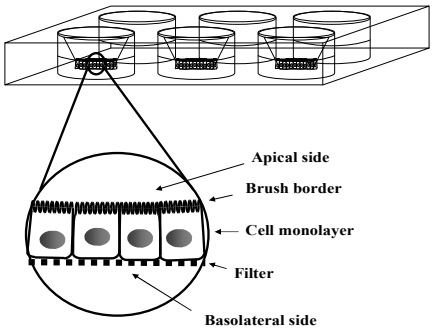

Figure 10. This figure shows a six well plate with six filter inserts and the cells with the brush border. (P Aspenström).

4.1.2 FVB male mice (Paper IV)

The influence of oleic acid on the absorption of MXR was tested in FVB male mice. BCRP is expressed in organs important for absorption (small intestine, colon), distribution (the blood-brain and placental-barriers) and elimination (canalicular membranes of hepatocytes) and also in kidney in mice. BCRP was shown to be expressed in the intestine of FVB mice (Enokizono *et al.*, 2007b; Jonker *et al.*, 2000). The highest amount of BCRP/villus was found in the ileum followed by jejunum, duodenum and colon (Han & Sugiyama, 2006; Tanaka *et al.*, 2005). The FVB mice have been used to study BCRP efflux of topotecan, a typical BCRP substrate (Yamagata *et al.*, 2007b). Furthermore, oral absorption of the BCRP substrates aflatoxin B1 and heterocyclic amines (IQ and Trp-P-1) was increased when given to a mice strain devoid of BCRP ($BCRP^{-/-}$ FVB male) compared to FVB Wt (Wild type) male mice (van Herwaarden *et al.*, 2006). Thus, the FVB mice strain has been shown to be a good model for studies of oral absorption of BCRP substrates. The amount of BCRP was slightly higher in male mice and therefore it seemed reasonable to choose male mice in the studies.

FVB Mice (NCrl, male, 8 weeks) were randomly assigned to different cages and acclimatized in a temperature- and humidity-controlled room with a 12 h light/dark cycle for one week before experiments were initiated. Tap water and standard diet were given free. The standard diet for the mice comprised of 7.43 E% fat and 0.88 w/w % MUFA and 0.88 w/w % PUFA. Mice were fasted over night with free access to water before the experiments.

4.2 Experimental procedure

4.2.1 In vitro absorption and accumulation experiments (papers I-III)

Absorption and accumulation experiments were performed at 37ºC in a cell incubator with continuous shaking (rpm 33), in order to minimize the influence of the aqueous boundary layer on the cell surface, which otherwise can influence permeability of substances (Artursson *et al.*, 1993).

Fatty acid emulsions of protonated fatty acids were prepared in experimental medium by sonification in Labsonic 1510 (B. Braun), maximum 400 W for 2 x 20 seconds, directly before addition of radiolabelled substances and before test emulsions were added to the cell monolayers. The fatty acid concentration of the test emulsions was not measured after sonification, but it was assumed that there was no loss of added fatty acid in the test emulsions. The pH of all emulsions was measured before added to the monolayers. The experiments were performed in six well plates and exposure was only carried out from the apical to the basolateral side of the monolayers. In all experiments the test solutions, with or without fatty acids, were added to the apical donor side and pure experimental medium was added to the basolateral receiver side of the Caco-2 cell monolayers.

As indicators of cell toxicity lactate dehydrogenase (LDH) and protein content in wells were measured in all cell studies. Cell integrity was determined by measuring transepithelial electrical resistance (TEER).

Paper I:

In experiment I, the influence of fatty acids, with or without Ca^{2+} in the test emulsion, on absorption of mannitol across Caco-2 cells were investigated. Emulsions of oleic acid (5, 15 or 30 mM) or DHA (5, 15 or 30 mM) were tested. To each well in the control groups and fatty acid exposed groups ^{14}C-mannitol was added in a concentration of 16 µM mannitol corresponding to 2 µCi/well. The test emulsions were added to the apical side of the monolayers (n=5-6 for each group) and samples were collected both basically and apically at different time points. Morphology of monolayers was analyzed with fluorescence microscopy, as described below, 4.3.4.

In experiment II, Caco-2 cell monolayers were exposed to Cd solutions (250 nM), with ^{109}Cd (1.5 mCi/well) as a tracer, in test emulsions of oleic acid (5 and 30 mM) or DHA (1 and 5 mM). Control groups were exposed to the Cd solution in experimental medium. Samples were collected at different timepoints and accumulation of Cd within cells was analyzed as described below, 4.3.3. The lipophilicity of Cd in oleic acid and DHA emulsions was measured as the octanol/water partition coefficient as described in 4.3.5.

Paper II:

The influence of fatty acids (DHA and oleic acid) on mannitol and Al absorption across Caco-2 cell monolayers, from the apical to the basolateral side, was investigated. To begin with, in order to find a concentration of Al that was not toxic to the cells, a dose-finding study was performed. Absorption of Al and ^{14}C-mannitol across Caco-2 monolayers was measured at different concentrations of Al (0, 1, 2, 4, 10, 20 and 30 mM) during 90 min.

Secondly, monolayers were exposed to ^{14}C-mannitol (2 μCi/well, corresponding to 16 μM mannitol) in emulsions of oleic acid (15 and 30 mM) or DHA (5 and 10 mM) and 2 mM Al. Samples for analyses of ^{14}C-mannitol and Al were collected after 90 min both from the apical and basolateral side of the monolayers. The morphology of cell monolayers were analysed with transmission electron microscopy (TEM), as described below, 4.3.4.

Paper III:

The influence of oleic acid on MXR absorption, restricted by BCRP, was investigated *in vitro* in the Caco-2 cell monolayer model.

Before the fatty acid experiments, cytotoxicity and apical to basolateral absorption of MXR were tested at different concentrations of MXR (0.1, 1, 10, 50, 100, 500 and 1000 μM MXR). Then, the effect of oleic acid (0.5, 1, 2, 4 and 5 mM) on the absorption of MXR (1 μM) across the cell monolayers was investigated. Radiolabelled ^{3}H-MXR (~200 000 cpm/well corresponding to 0.1 μCi) was used as a tracer. The influence of the BCRP substrate cimetidine (0.1 mM) and the BCRP inhibitor Ko143 (1μM) on MXR absorption over the monolayers was also tested. Moreover, the influence of oleic acid on the absorption of MXR after preincubation with oleic acid in culture medium (DMEM) for 5.5 h was tested. The incubation period was followed by a stepwise removal of oleic acid before MXR exposure. Quantitative gene expression of BCRP was measured by qRT-PCR and the amount of BCRP was measured with Western Blot after exposure to oleic acid during 3, 6, 9, 24 hours, as described below, section 4.3.6.

4.2.2 *In vivo*, absorption experiments (paper IV)

Paper IV:

Firstly, the influence of oleic acid on the absorption of MXR *in vivo* in mice was investigated.

A time-dependency study was carried out. Mice were dosed by gavage with an oleic acid emulsion (2.4 g/kg bw) with 1 mg MXR/kg bw and radiolabelled ^{3}H-MXR as a tracer. The dose was divided by three and given in 30 min

intervals during 1 h. The mice were sacrificed after 30, 60, 90 and 120 min post-dosing in groups of 5 to 6 individuals. Each oleic acid-treated group (sacrificed at different time points) had its own control group treated with MXR solution devoid of oleic acid.

Secondly, a dose dependency study was carried out, where mice were treated with emulsions of 0.6, 2.4 or 4.8 g /kg bw oleic acid and 1 mg/kg bw MXR /mouse. The dose were divided by three and given in 30 min intervals, as in the previous study. At 90 min post-dose the mice were sacrificed randomly.

In both experiments, mice were anaesthetized with isoflurane and sacrificed by heart puncture during blood sampling. Tissue samples from the liver, kidney, brain and intestine were removed and put in vials for dissolution, bleaching and scintillation counting as described below (4.3.2). Gene expression of BCRP was measured in the samples from jejunum, kept in RNA later, in the time-dependency experiment as described below (4.3.6).

4.3 Experimental techniques

Most experiments were carried out at the National Food Agency, Uppsala. Morphology studies with fluorescence microscopy were done at the Ludwig Institute for Cancer Research, Uppsala. Morphology studies with transmission electron microscopy, as well as gene- and protein-expression studies, were performed at the Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala, Sweden.

4.3.1 Cell integrity (papers I-III)

Trans-epithelial electrical resistance (TEER)

TEER is an indicator of epithelial integrity measuring ion flow through the cell monolayers. Electrical conductivity is mainly limited to the paracellular ion flux in Caco-2 cell monolayers, and therefore serves as a good marker for epithelial integrity, i.e. the tightness of TJs. Leaky TJs results in a lower TEER than in tight TJs. TEER was measured in an EndOhm 24 Snap chamber using an epithelial volthometer (EVOM24 mm, World Precision Instruments, WPI Inc. Stevenage, UK). TEER was measured as Ω x cm^2, and was typically 200 to 300 Ω x cm^2. The TEER value from an empty well was subtracted from the TEER value. TEER was expressed as

$(TEER_{\text{after preincubation with pure experimental medium}}/TEER_{\text{at the end of exposure}}) \times 100$

4.3.2 Cytotoxicity (papers I-III)

Cytotoxicity was measured as lactate dehydrogenase (LDH) leakage and protein content in the wells.

Lactate dehydrogenase leakage (LDH)

LDH is an intracellular enzyme present in most cells. If LDH is found in the cell medium it is an indication of that there is a cell leakage caused by a damaged cell membrane or lysis of the cells. LDH was measured using a cytotoxicity kit from Roche with a Caco-2 cell monolayer treated with 2% Triton X-100 as an indicator for maximal cell leakage of LDH. The samples were analyzed with spectrophotometry. LDH leakage for a sample was expressed as

$(LDH_{\text{leakage from exposed monolayers}}/LDH_{\text{leakage from TritonX- treated monolayers}}) \times 100$.

Protein content in wells

Protein content in wells is a marker of cell detachment from filters and of an increased leakage of intracellular proteins from the cells. An increase in protein content could hypothetically be due to an induction of protein synthesis. A low content of protein in the wells shows that cells have died or that cells have detached from the monolayer. Cells on filters were lysed with NaOH at the end of the experiments and then stored in -20°C before analyses. Protein analyses was performed with a photometrical method according to Hartree (Hartree, 1972) in paper I or a colorimetric assay kit (DC protein assay (BioRad, Life Science Research, Hercules, USA) in paper II, III.

4.3.3 Preparation and analysis of tested substances (papers I-IV)

Mannitol (paper I-II)

Radiolabelled ^{14}C-mannitol was analyzed in a β-scintillation counter (Wallac 1409, LKB Wallac, Finland). Scintillation liquid (Quicksafe A, Zinsser Analytic) was added to the samples that were equilibrated over night before scintillation counting. The apical to basolateral passage of ^{14}C-mannitol through monolayers was calculated as the percentage of the amount added at the apical side retrieved on the basolateral side at different timepoints or at the end of treatment.

Cadmium absorption over the monolayers and cadmium accumulation inside cells (paper I)

Radiolabelled Cd (^{109}Cd) was added to unlabeled Cd to a final concentration of 250 nM Cd (1.5 mCi/well). Radiolabelled ^{109}Cd was analyzed in a gamma counter (Nuclear Chicago, Model 1185, with a counting efficiency of 37%). Absorption of Cd was calculated as per mille of Cd that had passed across the monolayers from the apical to the basolateral side.

Accumulation of ^{109}Cd within cells was measured at the end of the experiments. Cells were washed on the apical side with EDTA twice to remove surface-bound Cd and thereafter cells were lysed with NaOH. Accumulation of Cd in the cells at the end of the experiment was calculated as the amount of ^{109}Cd in the cells (pmol Cd/mg protein for each monolayer).

Aluminium

The Al solution was prepared by mixing 100 mM Al chloride ($AlCl_3*6H_2O$) in experimental medium with 200 mM tri-sodium-citrate. Al was analysed using a Perkin-Elmer Analyst-600 atomic absorption spectrometer (Perkin-Elmer, Norwalk, Connecticut, USA) equipped with Zeeman background correction, transversely heated graphite furnace and an auto sampler AS-800. The limit of detection (LOD) determined for this method is 2 µg/l. Apical to basolateral absorption of Al across the cell monolayers was calculated as fractional absorption, i.e. the % of apical Al retrieved at the basolateral side of the monolayers.

MXR

In vitro studies: A stock solution of MXR in HBSS was prepared. Experimental solutions were prepared by dilution of the stock solution. Radiolabelled ^{3}H-MXR, was added as a tracer to the unlabelled solutions just before experiments, in order to get the final solutions for the oleic acid experiments of 1 µM MXR and for the dose-response experiment of 0.1, 1, 10, 50, 100, 500 and 1000 µM MXR. Radiolabelled ^{3}H-MXR were measured in the scintillation counter (Wallac 1409, LKBWallac, Finland). Scintillation liquid (Quicksafe A, Zinsser Analytic) was added to the samples that were equilibrated over night before scintillation counting. Apical to basolateral absorption of MXR across the cell monolayers was calculated as fractional absorption, i.e. the % of apical ^{3}H-MXR retrieved at the basolateral side of the cell monolayers.

Accumulation of ^{3}H-MXR within cells was measured at the end of the experiments. Cells were washed to remove surface-bound MXR as described for Cd accumulation above and thereafter the cells were lysed with NaOH. Scintillation liquid was added to sample vials as described previously. Accumulation of MXR in the cells was measured at the end of the experiment as pmol MXR/mg protein for each monolayer.

In vivo studies: A stock solution of MXR in HBSS was prepared. Each mouse was dosed with 1 mg MXR/kg bw as well as 1µCi ^{3}H-MXR to each mouse, as a tracer. Blood was sampled in heparinized tubes and plasma was separated by centrifugation. Samples from plasma, liver, kidney, brain and

intestine were dissolved and bleached for scintillation. Scintillation liquid was added to the samples and they were measured as described above. Results were expressed as cpm/g tissue or cpm/ml plasma. The ratios between tissues and plasma were calculated.

4.3.4 Morphological examination (papers I and II)

Fluorescence microscopy (paper I)

Morphology of cell monolayers was studied with fluorescence microscopy. The β-catenin was stained in cell-cell contacts and visualized. Cell monolayers on filters were fixed in para-formaldehyde in PBS, followed by permeabilisation with Triton-X and incubation with fetal bovine serum. The filters containing the cells were removed from the cell culture inserts with a scalpel and then incubated with a mouse monoclonal anti β-catenin antibody, followed by a tetramethyl rhodamine isothiocyanate (TRITC)-conjugated anti-mouse antibody, with a washing step between. The membranes were mounted on object slides using Fluoromount-G, and covered by a coverslip. Confocal analysis was performed on a Zeiss Axiovert 200 M equipped with a LSM 510 laser.

Transmission electron microscopy (paper II)

The morphology of cell monolayers was studied by using transmission electron microscopy (TEM). Monolayers were fixed in sodium phosphate buffer (SPB) containing glutaraldehyde, followed by fixation in SPB, containing 1% osmium tetra oxide and then finally embedded in Agar 100 resin (Agar Scientific, UK). The embedded cells were sectioned and stained with uranyl acetate and lead citrate (Lindmark *et al.*, 1998b). Sections were analyzed in a Philips 420 electron microscope operated at 60 kV. The magnification was 11000 times.

4.3.5 Octanol/water partition coefficients (paper I)

The octanol/water partitioning coefficient (P) is an indicator of the lipophilic nature of a substance. Octanol is assumed to have the lipophilicity comparable to a cell membrane and P describes the ability of a substance to partition in the lipophilic phase (Artursson *et al.*, 2001). The pH 7.4 was used in our experiments as it is the physiological pH of the interstitial fluid in microvilli of the small intestine. P of Cd (250 nM ^{109}Cd-solution) was tested at different concentrations of oleic acid (5 and 30 mM) and DHA (1 and 5 mM) according to the OECD Guidelines, test 107, with some modifications (OECD). The samples were measured in a gamma-counter and P was calculated as P = cpm (octanol)/cpm (Cd emulsion). The results were presented as logP.

4.3.6 Gene expression (papers III and IV)

Gene expression of BCRP was analysed in Caco-2 cells and in the intestine from mice exposed to oleic acid. Caco-2 cell monolayers (n=3) were exposed to oleic acid at different time-points. Each time-point had its own controls (n=3). In addition, samples from intestine (jejunum) from oleic acid exposed mice were analysed. Total RNA was isolated by using a Nucleospin®RNAII kit from Macherey-Nagel (Germany) according to the manufacturer's instructions. The integrity of the isolated RNA was confirmed by ethidium-bromide agarose gel electrophoresis. Quantification of the RNA was performed according to the RNA-specific RiboGreen protocol with Dnase I (In vitrogen, Stockholm, Sweden).

BCRP gene expression was measured by real-time RT-PCR, using the QuantiTect RT-PCR kit with SYBR Green (Qiagen®, Inc, Valencia, CA, USA) according to the instructions by the manufacturer and the primers in table 3. RT-PCR reactions were performed by the use of a Rotor-Gene, RG 3000 instrument (Corbett Research, Mortlake, Australia) in the presence of 200 ng templates RNA. Melt curve analyses were performed for each sample in direct connection to the RT-PCR. For studies of the specificity of the obtained PCR products Rotor Gene software was applied.

Table 3. *Primer sequences used for quantitative real-time PCR*

Primer	Oligo sequence
BCRP forward	5´-GCT TAG ACT CAA GCA CAG CAA A-3´
BCRP reverse	5´-GGC CCG TGG AAC ATA AGT C-3´
mBCRP forward	5´-CGC AGA AGG AGA TGT GTT GA-3´
mBCRP reverse	5´- TTG GAT CTT TCC TGG CTG CT-3´

In the cell experiments the relative quantification of BCRP gene expression was calculated. Calculated values describe the change in expression of the target gene in the exposed groups in relation to the expression in the untreated controls using the $2^{-(\Delta Ct)}$ method (Schmittgen & Livak, 2008).

In the *in vivo* experiments, BCRP was quantitatively measured. A standard curve for each real-time RT-PCR was prepared, and concentration of BCRP in each sample from the intestine was calculated from the standard curve.

4.3.7 Protein expression (paper III)

Western blot analyses were done to measure the amount of BCRP in Caco-2 cells, which were exposed for oleic acid (1mM) in culture medium for 1.5 h or 6 h. Monolayers of Caco-2 cells only exposed to DMEM were used as controls.

After exposure, cells were scraped off the filter inserts and subsequently lysed in hypotonic lysis buffer, sonificated and centrifuged to remove insoluble material. The protein content in the cell lysate was measured as described above (4.3.2). Protein (15 μg) was unfolded by using Laemmli buffer with SDS and boiling, followed by separation on SDS-PAGE. After the electrophoresis, protein was transferred to nitrocellulose membranes (BioRAD laboratories). Membranes were blocked in blocking agent and then incubated with BCRP/ABCG2 antibodies (BXP-53, Abcam) diluted in PBST followed by the secondary antibodies HEP conjugated rabbit polyclonal anti-rat antibodies (1Ab 6374, Abcam) diluted in PBST. BCRP was detected by using enhanced chemi-luminescence (General Electric Healthcare). Detection and quantification of the intensities of the obtained 72 kD bands were done with the Chemi-Doc Gel Quantification System and Quantity One software (BioRad). The protein expression of BCRP was normalized against the protein expression of tubulin.

4.4 Statistical analysis

The *in vitro* experiments were carried out using 3 to 6 cell monolayers per exposure group. Results were expressed as mean or median ± S.D. Differences between the control and exposed groups were tested using the Mann-Whitney U-test. Mann Whitney U-test is a two sample test, testing the hypothesis that the control and the exposed groups have equal medians, against the possibility that the two groups do not have equal medians (a two-sided test). The level of significance was set at 0.05. The correlations between fractional Al and mannitol absorption were analyzed using the nonparametric Spearman rank correlation test and was expressed as correlation coefficients. All statistical analyses were performed using Graph Pad Prism 5.00 or Minitab 15.

5 Results and discussions

This section contains the results from this thesis and a few additional results that have not yet been published. For the complete description of each studies the individual papers could be considered, paper I-IV.

5.1 Influence of passage number on absorption of aluminium and mannitol

Caco-2 cells are grown in many laboratories under different conditions such as various culturing techniques, seeding densities, culturing time and different passage numbers, resulting in quite heterogeneous cell populations (Hayeshi *et al.*, 2008). Thus, the general population of Caco-2 cells consists of a heterogeneous mixture of cell clones. Each passage might result in a selection of fast growing subpopulations (Yu *et al.*, 1997). Comparison of results from different laboratories may therefore be difficult. Passage numbers ranging from 20 to over 100 have been used in different studies (Delie & Rubas, 1997).

In an unpublished experiment, we elucidated the importance of passage number (50 to 100) of cultured cells on absorption of aluminium and mannitol. The cells at different passages were fully differentiated on filters and transport experiments were performed as described for Al and mannitol studies in paper II. The cells were exposed apically to 0, 5 or 25 mM Al-citrate and ^{14}C-mannitol (unpublished data). It was found that the relative TEER (TEER before exposure compared with TEER at the end of exposure) increased with increasing passage number up to passage 100, especially in the Al-exposed groups. Thus, the epithelium was tighter at higher passage numbers, which resulted in a decreased absorption of mannitol and Al with increasing passage number. Mannitol absorption decreased both in control and Al exposed monolayers at increasing passage numbers. Similarly, it has been shown that passage-dependent absorption of mannitol was inversely associated with

passage-dependent changes in TEER (Briske-Anderson *et al.*, 1997; Yu *et al.*, 1997). However, the results indicated that the number of passages in serial culturing of Caco-2 cells has an impact on the sensitivity of the Caco-2 cell monolayers for Al. Al exposure caused a more pronounced decrease in relative TEER at lower passage numbers, suggesting a higher Al sensitivity at lower passage numbers than at higher passage numbers.

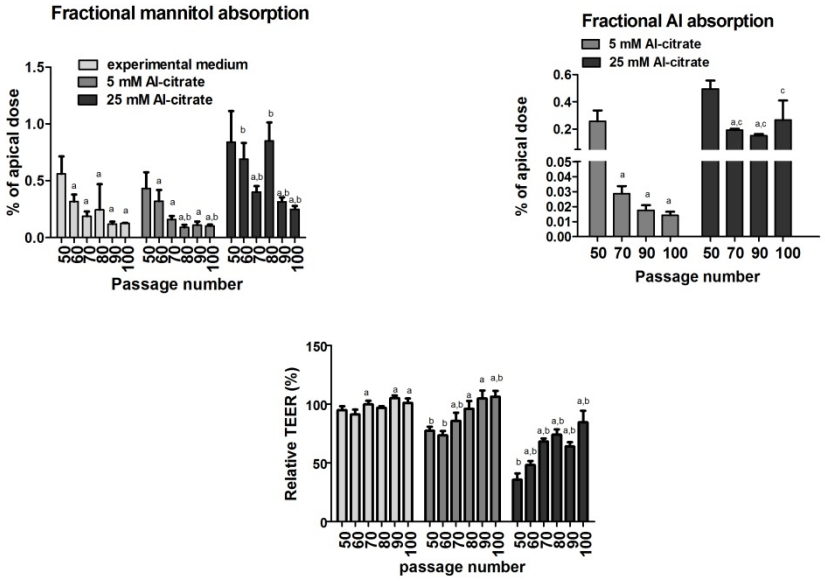

Figure 11. The influence of different passage numbers (50, 60, 70, 80, 90 and 100) on the absorption of ^{14}C-mannitol and Al across fully differentiated Caco-2 cell monolayers after 60 min exposure to ^{14}C-mannitol in Al-free experimental medium, or 5 or 25 mM Al-citrate in experimental medium, (n=6) (mean $\pm$SD). [a)] Statistically significant in relation to passage 50 in each exposed group, at $P<0.05$ (Mann Whitney U-test). [b)] Statistically significant compared with each corresponding passage in the control group, $P<0.05$. [c)] Statistically significant compared with each corresponding passage in 5 mM Al-citrate at $P<0.05$ (Mann Whitney U-test).

Increased growth rate, TEER and sucrose activity in Caco-2 cells were found as passage increased (Briske-Anderson *et al.*, 1997). In addition, more homogenous cells were found at confluence at higher passages. This indicate that a selection process might be possible, where more robust cells have survived. Higher passage number was also shown to increase the possibility that cell monolayers grows in multiple layers causing an increased TEER (Briske-Anderson *et al.*, 1997). However, we have not found multiple layers in our experiments. Thus, it is important to follow the same procedures from time to time in a laboratory and also to use the same passages to get comparable

results. We concluded that it is appropriate to use high passaged cell monolayers to reduce the impact of toxicity on the cells, influencing absorption.

5.2 Fatty acids influence tight junction permeability and absorption (papers I and II)

5.2.1 Absorption of mannitol and cadmium across Caco-2 cell monolayers is enhanced by DHA and oleic acid (paper I)

The influence of fatty acids (oleic acid and DHA) on the absorption of mannitol and Cd in Caco-2 cell monolayers was investigated. Apical to basolateral absorption of mannitol across the cell monolayers increased with increasing concentrations of both oleic acid (15 and 30 mM) and DHA (5, 15 and 30 mM) in the presence of Ca^{2+} after 90 min exposure (figure 12 A). When the experimental medium was devoid of Ca^{2+} the effect was approximately 7 times higher for DHA and 3-4 times higher for oleic acid (figure 12 B). At the same time TEER decreased in the same manner (figure 12 C). DHA was more cytotoxic than oleic acid, but the effects were still dependent on the presence of Ca^{2+} in the experimental medium.

The results show that the presence of Ca^{2+} in the experimental medium is important for the function of TJs in Caco-2 cell monolayers, as TEER decreased and cytotoxicity of fatty acids increased when experimental medium was devoid of Ca^{2+}. In presence of Ca^{2+} the fatty acids had effects on tight junctions without causing measurable cytotoxicity (Aspenstrom-Fagerlund *et al.*, 2007). It is important to keep the Ca^{2+} level in medium at a physiological level on both sides of the cell monolayers (Anderberg *et al.*, 1993). TJs between intestinal epithelial cells are dynamic and the TJ tightness can be modulated by intracellular and extracellular events (Delie & Rubas, 1997).

The TJ protein β-catenin is important for the function of cadherin and its adhesive properties. In paper I, the morphology of cell monolayers was studied with fluorescence microscopy. We showed that control monolayers exposed to experimental medium with or without Ca^{2+} for 90 min had a distinct localization of β-catenin to the cell-cell contacts and a homogenous and confluent cell monolayer (figures 13 A and C).

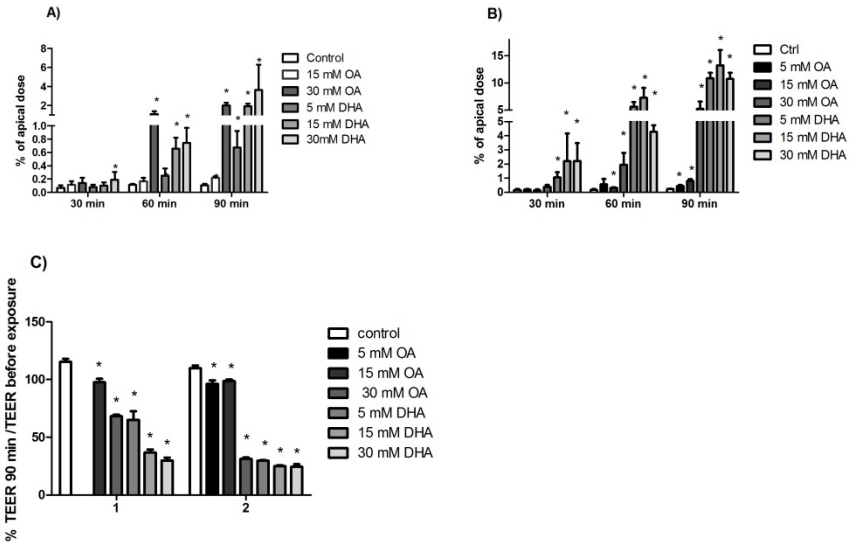

Figure 12. Absorption of ^{14}C-mannitol in Caco-2 cell monolayers exposed to emulsions of oleic acid (OA) or DHA in experimental medium **A**) with or **B**) without Ca^{2+} and Mg^{2+} for 90 min. **C**) Relative TEER in % for Caco-2 cell monolayers exposed for 90 min to oleic acid or DHA, in experimental medium (1) with or (2) without Ca^{2+} and Mg^{2+} (n=6). *)Statistically significant in relation to controls. $P<0.05$ (Mann- Whitney U-test).

Cells exposed to 30 mM oleic acid for 90 min in experimental medium with Ca^{2+} still showed confluent monolayers. However, an increased dissolution of the cell-cell contacts was seen as the localization of β-catenin was not as distinct compared to controls (figure 13 B). Cells exposed to 5 mM DHA in experimental medium without Ca^{2+} showed a profound alteration of cell-cell contacts (figure 13 D). In addition, the thickness of the cell monolayers was decreased. The results, visualized by fluorescence microscopy, confirmed the results that oleic acid and DHA even at rather low concentrations (5 mM) have cytotoxicity effects in Caco-2 cell monolayers. In all further studies experimental medium with Ca^{2+} was used as the cytotoxicity was more pronounced when experimental medium was devoid of Ca^{2+}.

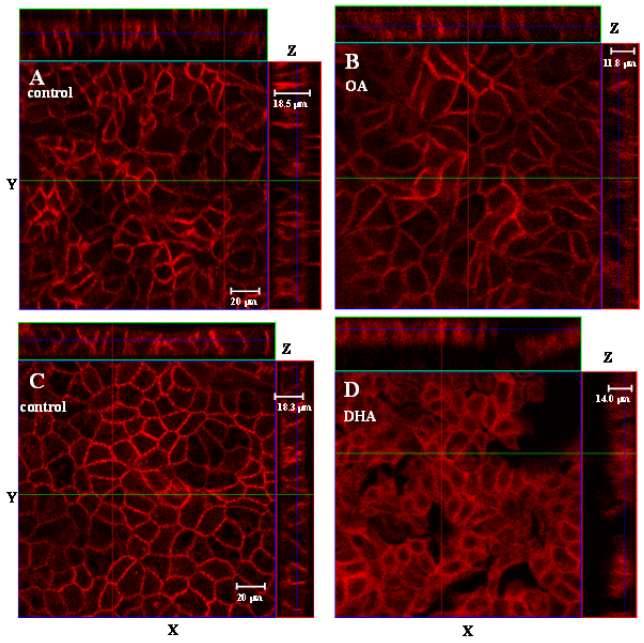

Figure 13. The organization of cell-cell contacts was visualized by staining cells with a mouse anti-β-catenin antibody followed by TRITC- conjugated anti-mouse antibody. Control monolayers visualized in (A) and (C) was treated with experimental medium with and without Ca^{2+} and Mg^{2+}, respectively. (B) Cell monolayers were treated with emulsion of 30 mM OA in presence of Ca^{2+} and Mg^{2+}. (D) Cell monolayers were treated with 5 mM DHA in experimental medium devoid of Ca^{2+} and Mg^{2+}. X, Y and Z are the co-ordinate axis that shows the cells three-dimensionally. (Fluorescence microscopy: Photo P Aspenström)

In paper I, it was also investigated if oleic acid and DHA influence absorption and accumulation of Cd, which is a poorly absorbed hydrophilic toxic substance. In a dose finding study, Caco-2 cell monolayers were apically exposed for 180 min to different concentrations of $CdCl_2$ in experimental medium containing Ca^{2+}. It was found that a concentration of 250 nM $CdCl_2$ did not affect TEER or LDH leakage. In a subsequent experiment the effects of oleic acid and DHA on absorption of Cd from the apical to the basolateral side of Caco-2 cell monolayers was investigated. Absorption of Cd in emulsions of DHA (1 or 5 mM) increased after 120 and 180 min exposure, simultaneously as TEER and protein content in cell monolayers was decreased, and LDH leakage increased (figure 14).

An octanol/water partitioning experiment with Cd showed that the lipophilicity of Cd increased in the following order:
5 mM oleic acid < 30 mM oleic acid ≤ 1 mM DHA < 5 mM DHA

Thus, the lipid solubility of Cd increased in the presence of both oleic acid and DHA. Exposure to Cd in emulsions of oleic acid (5 and 30 mM) for 180 min, did however not result in an increased absorption of Cd. TEER, LDH leakage and protein content in cell monolayers were either not influenced. This suggests that increased lipid solubility was not the reason for the higher absorption of Cd in presence of DHA. It therefore appears that the paracellular absorption of Cd is increased when cells are exposed to DHA. However, as Cd is also absorbed through other pathways as the passive and active transcellular routes (Jumarie *et al.*, 1999), it could not be proven in these experiments that the influence of DHA on Cd absorption is on the paracellular pathway.

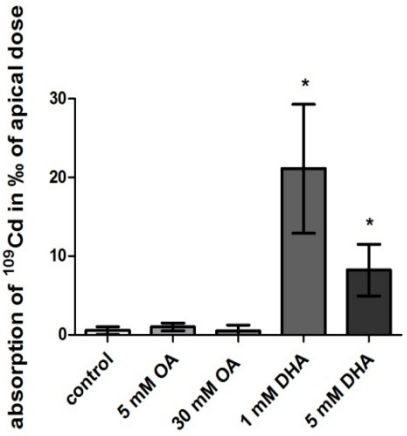

Figure 14. Absorption of Cd (‰ of apical dose), following exposure of Caco-2 cell monolayers to ^{109}Cd in emulsions of oleic acid or DHA during 180 min (n= 5).

DHA was shown to increase Cd absorption at the same time as it was toxic to the cell monolayers and caused leakier TJs. The disruption of the cell monolayers could be the mechanism behind the increased Cd absorption rather than effects on TJs. Mannitol absorption was increased by both oleic acid and DHA and correlated with the decrease in TEER, without any pronounced cell toxicity. Mannitol is known to be entirely absorbed through the paracellular route (papers I and II). The increased paracellular permeability of the intestinal epithelium caused by oleic acid and DHA is a factor to consider in risk assessment.

5.2.2 Aluminium and mannitol absorption across Caco-2 cell monolayers is increased by emulsions of oleic acid and DHA (paper II)

In this paper it was hypothesized that Al followed the same pattern of absorption as mannitol, i.e. the paracellular pathway, and also that fatty acids like DHA and oleic acid increase the absorption of Al and mannitol through the paracellular pathway in Caco-2 cell monolayers. In a dose finding study, the apical to basolateral absorption of Al in Caco-2 cell monolayers was investigated after exposure for 90 min. A dose-dependent (0, 1, 2, 4, 10, 20 and 30 mM Al) decrease in TEER starting at 2 mM Al was found, although the absorption of Al and mannitol did not increase significantly at concentrations up to 10 mM Al. At higher doses of Al (20 to 30 mM) the absorption increased, probably due to increased cell toxicity at these concentrations as evidenced by a considerably decreased TEER. A strong correlation between Al and mannitol absorption was found which confirms that Al similarly as mannitol is absorbed by the paracellular route. These findings are in line with previously published studies (Zhou & Yokel, 2005; Whitehead *et al.*, 1997; Provan & Yokel, 1988). For further experiments with Al and fatty acids a concentration of 2 mM Al was chosen, since this concentration of Al had negligible effects on the Caco-2 cell monolayers with regard of LDH leakage and TEER.

Emulsions of oleic acid (15 and 30 mM) and DHA (5 and 10 mM) increased the apical to basolateral absorption of Al and mannitol across Caco-2 cells in a dose-dependent manner. A relation was seen between TEER and absorption across Caco-2 cell monolayers, i.e. when TEER decreased the absorption after 90 min exposure was concomitantly increased (figure 15). TEER decreased in all groups exposed to emulsions of fatty acids and Al, but only marginally in the 2 mM Al group, compared to the group exposed to Al-free experimental medium. Al absorption was significantly increased in emulsions of 15 and 30 mM oleic acid and 10 mM DHA, while mannitol absorption was only significantly increased in emulsions of 30 mM oleic acid and 10 mM DHA. The results showed that TEER is decreased before Al and mannitol absorption is affected and without cytotoxic effects (fig 15 A and B). Thus, it is possible that pores between cells are only slightly affected by the fatty acids at low concentrations allowing passage of small ions without affecting Al and mannitol absorption. Another possible explanation is that TJs consists of pores with different sizes, and that only small pores are affected by exposure to low doses of fatty acids (Van Itallie *et al.*, 2008; Watson *et al.*, 2005; Watson *et al.*, 2001).

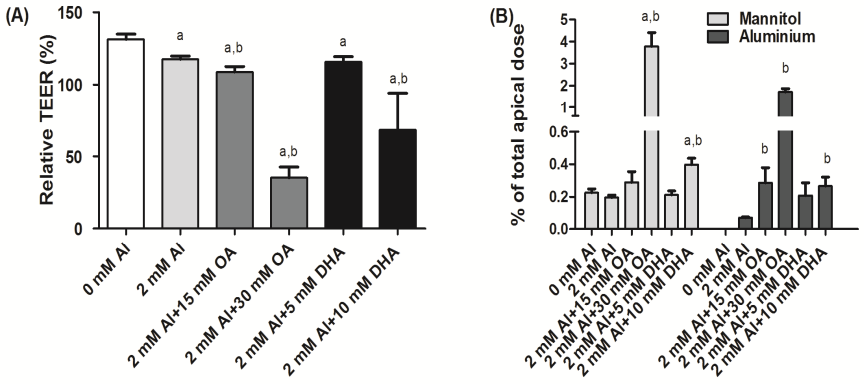

Figure 15. Absorption of Al in Caco-2 cell monolayers after exposure of 2 mM Al-citrate in emulsions of oleic acid (OA) or DHA for 90 min. **(A)** TEER at the end of exposure expressed as % of TEER before exposure. **(B)** Fractional absorption of Al and ^{14}C- mannitol expressed as % of apical dose (n= 6). [a)] Statistically significant in relation to Al-free experimental medium, P<0.05 (Mann Whitney U-test). [b)] Statistically significant in relation to 2 mM Al-citrate in experimental medium. P<0.05 (Mann Whitney U-test).

In morphology analysis with transmission electron microscopy (TEM) we found that cell monolayers exposed to Al-free experimental medium showed normal cell morphology with properly defined TJs and desmosomes (DS) (figure 16). Exposure of cells to emulsions of 30 mM oleic acid and 2 mM Al resulted in a less structured morphology of TJ and DS as well as of shorter microvilli. Cell-cell contacts were also less distinct and blurred. Thus, in this study our hypothesis that long-chained fatty acids compromise the TJs integrity causing an increased paracellular absorption of Al was supported.

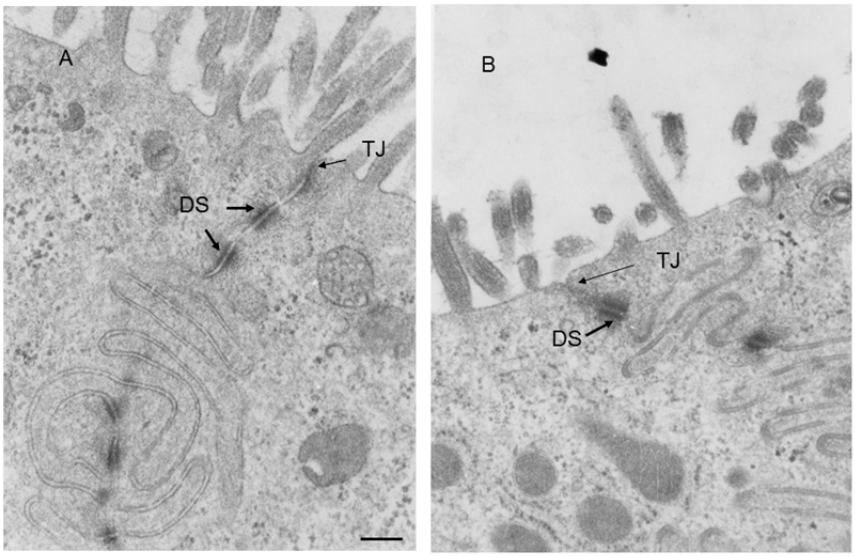

Figure 16. Morphological analysis of Caco-2 cell monolayers with transmission electron microscopy (TEM) showing a cross-section of a monolayer with apical microvilli, tight junction (TJ), desmosomes (DS) and other cell-cell contacts visible: (A) was exposed 90 min to Al-free experimental medium and (B) was exposed for 90 min to 2 mM Al-citrate in emulsion of 30 mM oleic acid. The bar represents 200 nm. (Electron micrograph: Tapio Nikkilä)

5.3 Oleic acid influence BCRP efflux of mitoxantrone, *in vitro* and *in vivo* (papers III and IV)

5.3.1 The effect of oleic acid on BCRP-mediated efflux *in vitro* in Caco-2 cell monolayers (paper III)

It was hypothesised that oleic acid influences the efflux function of BCRP and thereby increases the apical to basolateral absorption of MXR in Caco-2 cell monolayers. In a dose finding study it was found that absorption of MXR through the Caco-2 cell monolayers increased dose-dependently during 60 min exposure. The ratio between the MXR dose given apically and the amount retrieved on the basolateral side was not significantly different between groups, suggesting that there is no saturation of the efflux protein after exposure of MXR up to 1000 µM. No toxic effects on the monolayers were found, as measured by LDH and TEER. Thus, the cells tolerated rather high concentrations of MXR. However, 1 µM MXR was used in our Caco-2 cell experiments as this concentration has been used by other investigators and it is

also in the same range as concentrations used in other experiments for inhibitors like Ko143 (Hubatsch *et al.*, 2007; Matsson *et al.*, 2007; Yamagata *et al.*, 2007a).

Apical to basolateral absorption of MXR was increased with increasing concentration of oleic acid (0.5, 1, 2, 4 and 5 mM) and also by the BCRP substrate cimetidine (figure 17a). Toxicity, as indicated by LDH leakage was only found in the highest dose group. TEER was only slightly decreased in groups exposed to 0.5 to 4 mM oleic acid, but markedly so in the 5 mM group. The increased absorption in the 5 mM oleic acid treated group could be due to an increase in paracellular absorption. However, in paper I and II we showed that emulsions of 15 mM oleic acid containing Ca^{2+} did not increase absorption of mannitol, which is a paracellular marker. In addition, mannitol is a much smaller molecule than MXR and should therefore more readily be absorbed by the paracellular route. These previous findings strongly suggest that the effect of oleic acid on MXR absorption is due to the oleic acid effect on BCRP and not on the paracellular absorption.

The accumulation of MXR in the Caco-2 cells increases with increasing concentrations of oleic acid at doses of 2 mM or higher in our experiments. MXR absorption was also increased when cell monolayers were concomitantly exposed to MXR and the BCRP inhibitor Ko143, which further suggest that BCRP is involved in the restriction of MXR absorption in our Caco-2 cells (figure 17c).

It was further investigated if pre-incubation of Caco-2 cell monolayers with oleic acid for 5.5 to 6 h influenced the oleic acid-dependent effect on MXR transport. In this experiment oleic acid was successively removed during and before the final MXR absorption experiment. Results showed that the increased absorption of MXR was not mediated by the presence of the oleic acid emulsion, since an increased MXR absorption persisted even after removal of the oleic acid emulsion (results not shown).

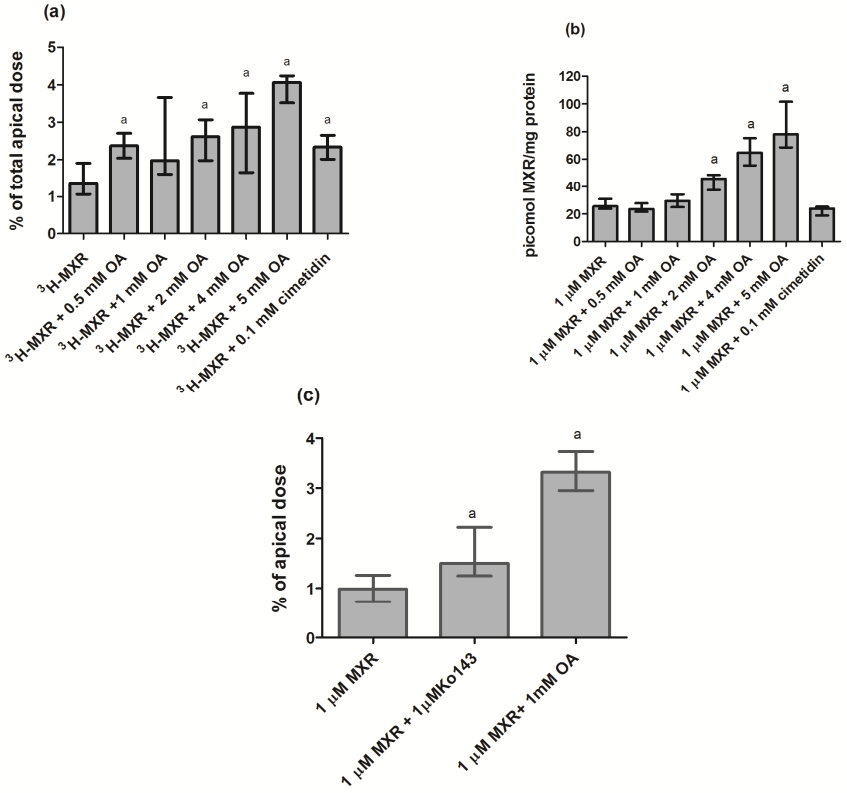

Figure 17. Apical exposure of Caco-2 cell monolayers in (a) and (b) to 1 μM MXR in emulsions of 0, 0.5, 1, 2, 4 and 5 mM oleic acid (OA) or 1 μM MXR and 0.1 mM cimetidine for 60 min. (a) MXR retrieved on the basolateral side, expressed as % of apical dose (median ± range) and in (b) MXR accumulated in cells after 60 min exposure, expressed as picomol/mg protein (median ± range). (c) Cells were exposed to 1 μM MXR , 1 μM MXR and 1 μM Ko143 or 1 μM MXR in emulsion of 1 mM oleic acid for 60 min and MXR was retrieved on the basolateral side, expressed as % of apical dose, n=5-6, median ± range. a) Statistically significant difference in relation to the group exposed to 1 μM MXR, $p<0.05$ (Mann-Whitney U-test).

It was also investigated if oleic acid had any influence on the gene expression of BCRP in Caco-2 cell monolayers. Results showed that oleic acid (1 mM) in culturing medium caused a significantly increased gene expression of BCRP after 6 hours exposure of Caco-2 cell monolayers (figure 18A). No effect could be seen after 1.5 h but after 3 and 9 h there was a tendency of increased gene expression due to the oleic acid exposure (figure 18A). However, Western blot quantification of the BCRP protein in the control group and the 1.5 and 6 h exposed groups did not show any differences between the groups (figure 18B).

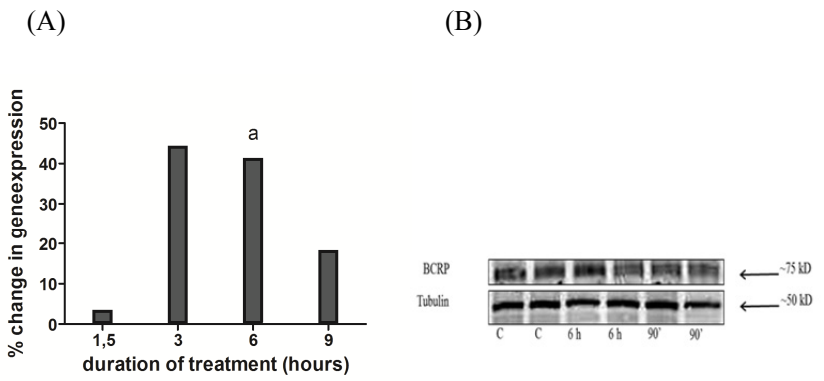

Figure 18. (A) BCRP gene expression in Caco-2 cell monolayers exposed apically to 1 mM oleic acid for 1.5, 3, 6 or 9h in comparison with control monolayers exposed to DMEM for each time point. (B) Western blots of BCRP and tubulin in Caco-2 cell whole protein lysate. Control (C), Caco-2 cells treated with oleic acid (OA) for 90 min (90') or 6 h, (n= 2-4). a) Statistically significant difference in relation to the controls at each time point, $p<0.05$ (Mann-Whitney U-test).

Taken together, the findings that oleic acid increases MXR absorption and the gene expression of BCRP, implicate that the effect of oleic acid on the transport of MXR is mediated by an inhibition of the function of the BCRP transporter.

5.3.2 *In vivo* studies on mice (paper IV)

The findings in paper III that oleic acid most probably inhibit the function of BCRP implicate that this also could happen *in vivo*. As a first step to verify these findings *in vivo* studies were carried out in male FVB mice. Oleic acid caused significantly increased levels of MXR in plasma, liver, kidney and brain at different time points (60 to 120 min) after the last oral dose of MXR in emulsions of oleic acid, when compared to respective time control (figure 19). The retention of MXR in the duodenum and upper part of jejunum was increased at 60 min after the last dose compared with controls.

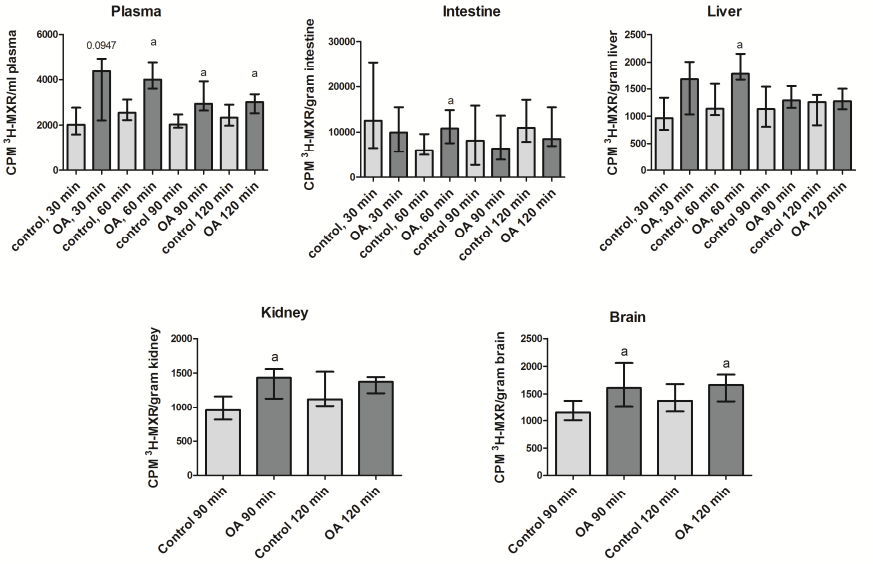

Figure 19. Time dependency of MXR levels in tissues from male mice (FVB NCrl) treated orally with MXR and ^{3}H-MXR in emulsions of oleic acid. Samples from plasma, intestine, liver, kidney and brain were analyzed for ^{3}H-MXR, (n= 5-6). a) Statistically significant differences in relation to the timed control group exposed to 1 μM MXR, $p<0.05$ (Mann-Whitney U-test).

Furthermore, in a second experiment, levels of MXR were significantly increased in plasma, kidney and brain at all tested doses of oleic acid (0.6, 2.4 and 4.8 g /kg). In liver MXR levels increased only after dosing of 2.4 g oleic acid/kg. In the duodenum and upper part of the jejunum, MXR levels increased only after 0.6 g oleic acid/kg (figure 20).

However, no dose response was seen. This is in agreement with studies performed with surfactants like Pluronic 85 and Tween 20 given to mice simultaneously as the BCRP substrate topotecan, where the middle dose gave a more pronounced effect than the highest dose (Yamagata *et al.*, 2007b). These authors suggested that surfactants may saturate the efflux pump competitively and thereby inhibit substrate binding to the efflux pump. Higher concentrations will thus not cause any further inhibition.

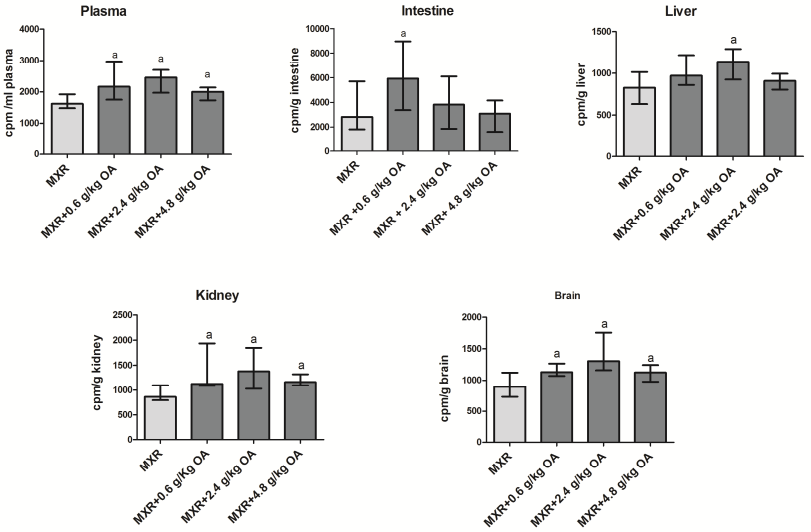

Figure 20. Dose-dependency of MXR absorption in tissues from male mice (FVB NCrl) treated orally with MXR and ^{3}H-MXR in emulsions of oleic acid. Samples from plasma, intestine, liver, kidney and brain were analyzed for ^{3}H-MXR, (n= 6). a) Statistically significant differences in relation to the group exposed to 1 μM MXR, $p<0.05$ (Mann-Whitney U-test).

Several studies have shown that surface active substances, like cremophor, PEG esters, polysorbates (e.g.Tween 20 and 80), polymers (e.g. Pluronic P85) similarly as oleic acid, negatively affects the function of BCRP and thereby increase absorption of BCRP substrates in the intestine (Hirunpanich & Sato, 2009; Wempe *et al.*, 2009; Constantinides & Wasan, 2007; Yamagata *et al.*, 2007b).

The increased absorption of BCRP substrates such as MXR, caused by oleic acid, might be due to several alternative mechanisms such as a) increased lipid solubility of the substrate, b) negative effects on the barrier function of TJs, c) direct influence on the function of BCRP, or d) indirect effects on its function, due to for instance alterations in the cellular membrane around the transporter or e) alterations in the energy availability for the transporters. In vitro (paper III), it was shown that oleic acid-induced increases in absorption of MXR were not due to an increased paracellular MXR absorption or increased lipophilicity of MXR. Thus, an effect on the BCRP is the most likely explanation to our observations.

The ratio between plasma and tissue MXR concentrations in mice did not differ between the different time groups or the different dose groups (paper IV). This means that the effect on brain, kidney and liver accumulation is solely dependent on the plasma level. The increased levels in the brain could,

in part, be due to the fact that the whole brain was sampled and not perfused, to get rid of residual blood in the tissue. However, when comparing the MXR levels in whole blood with the levels in the brain it was found that the brain contained more CPM than the blood on a volume/weight basis. Thus, the results suggest that the blood-brain barrier did not efflux MXR efficiently resulting in an increased uptake of MXR through the brain blood barrier when the blood MXR levels increased.

Gene expression analyses with RT-PCR showed in our mouse experiments that BCRP was expressed in the intestine. However, no significant differences in BCRP gene expression in samples from jejunum were found. Nevertheless, a tendency towards an increase by time in gene expression was observed in the intestine from mice sacrificed 120 min after cessation of oleic acid treatment. This finding might indicate that BCRP gene expression in mice is influenced by oleic acid exposure.

The dosing schedule used where oleic acid was given simultaneously as MXR three times during 1 h appeared to give robust results. In a study where oleic acid was given 30 min before MXR and the mice sacrificed 30 min after dosing, there was no increase of MXR in tissues or plasma (unpublished results). This suggests that the timing of contact between the intestinal epithelium and oleic acid and MXR is an important determinant for causing an effect.

On a bodyweight basis, the doses given to mice were 2 to 7 times higher than the amount of free oleic acid that humans normally are exposed to through the diet. Nevertheless, we found that the lowest dose, twice the average intake in humans, increased MXR concentrations in plasma, brain, kidney and intestine in mice. In addition, the passage time of food in mice is faster than in humans which means that humans will be exposed during longer time periods for oleic acid and in that way oleic acid have the ability to affect human BCRP for longer time periods.

6 Conclusions

The impacts of fatty acids common in food on the absorption of substances relevant in a food contaminant perspective, i.e. cadmium and aluminium, have been investigated. The marker substances mannitol and mitoxantrone was used to study different absorption pathways in the intestinal epithelium. Mannitol are commonly used in studies of the paracellular pathway and MXR in studies of the efflux protein BCRP, preventing substances to be absorbed by the active transcellular pathway.

DHA in physiologically relevant doses caused a significantly increased absorption of mannitol, Cd and Al through Caco-2 cell monolayers. Oleic acid, also in physiologically relevant doses, significantly increased the apical to basolateral absorption of mannitol and Al, but not Cd, through Caco-2 cell monolayers. TEER measured across the Caco-2 cell monolayer decreased as the doses of fatty acid increased. The strong relationship between changes in TEER and absorption of mannitol, Al and Cd clearly suggest that the increased absorption is due to increased paracellular absorption. Our findings were confirmed with morphological analyses with fluorescence microscopy and transmission electron microscopy. Thus, the paracellular absorption of poorly absorbed hydrophilic substances was found to be increased by oleic acid and DHA.

Oleic acid decreased BCRP mediated efflux of MXR in Caco-2 cell monolayers, causing an increased absorption of MXR across the monolayers. Oral exposure of oleic acid to mice caused increased levels of MXR in blood, brain, kidney, liver and intestine. In addition, oleic acid up-regulated the BCRP gene expression in Caco-2 cell monolayers. These findings indicate that fatty acids in food inhibit the function of BCRP and thereby increase intestinal absorption of its substrates. The increased gene expression *in vitro* may be a

compensatory reaction to the inhibition of BCRP mediated efflux. The effects of fatty acids on BCRP are important to consider in risk assessment of toxicants in food.

In risk assessment of food toxicants the oral bioavailability is often assessed in laboratory animals that are generally kept on low-fat feeds compared with the human diet. Our findings that food components like fatty acids increase oral absorption of substances by different intestinal epithelial pathways, is an important finding that ought to be considered in future risk assessment. Consequently, risk based limits may be underestimated if they are based on animal studies using diets with low fat content.

7 Future Perspectives

In paper I we found that DHA but not oleic acid caused an increased absorption of Cd through Caco-2 cell monolayers. These findings would be interesting to verify in vivo. In such a study, DHA could be given together with radioactive labelled Cd to mice using the same dosing schedule as in paper IV, i.e. three times during one hour. The absorption of Cd may take place by different pathways through the intestinal epithelium. This could in fact be the reason why oleic acid did not increase Cd absorption even though it was shown in paper II that absorption of Al and mannitol were increased by oleic acid. The impact of DHA on transporter proteins essential for Cd efflux or absorption could be interesting to study in Caco-2 cells and also in mice.

In paper II we showed that Al most probably was absorbed through the paracellular pathway and that oleic acid and DHA increased this absorption in Caco-2 cells. Whether this is true also *in vivo* can in fact be studied in humans. Al is included in antacid drugs used in humans. In an experiment, volunteers can be dosed with antacids containing Al according to the doses recommended for the medicine several times a day during 7 days, followed by sampling of urine and blood. After this dosing period a wash out period with no medication for 2 weeks can follow before sampling of blood and urine. After these two periods antacids containing Al could be given together with DHA in capsules 2 to 3 times a day during 7 days, followed by sampling of urine and blood to investigate if the Al levels are higher after dosing with DHA. This experiment might give an answer if our findings in the Caco-2 cells are applicable also for humans.

In paper III and IV we found that oleic acid increased absorption of the BCRP substrate MXR both in Caco-2 cell monolayers and in mice. The gene expression of BCRP was also increased by oleic acid exposure in Caco-2 cells.

This indicated that oleic acid impairs the function of BCRP which may cause a compensatory up-regulation of the gene. The mechanism for this effect is not known. As both PPARα and PPARγ nuclear receptors are activated by fatty acids and that it has been shown that BCRP is up-regulated by these receptors, it should be interesting to study the importance of oleic acid for the function of BCRP via its regulation through the PPARα and PPARγ nuclear receptors.

To finally support our hypothesis that fatty acids in food influence the oral absorption of BCRP substrates, studies in humans could be possible to do. Some BCRP substrates like folic acid and riboflavin are normally present in human tissues and are essential for many physiological processes in the human body. The influence of oleic acid on the absorption of folic acid could therefore be studied in humans. A similar procedure as for Al could be used.

Development of resistance to drugs used as antimicrobials and anthelmintics is an emerging problem in the world. Resistance to the anthelmintics benzimidazoles used for treatment of e.g. liver flukes (fasciola hepatica) in animals and humans is an increasing problem (Brennan *et al.*, 2007). Some of the benzimidazoles (triclabendazole, albendazole, oxfendazole) are known substrates and inhibitors of BCRP. Investigation of the effect of oleic acid on the efflux of benzimidazoles in worms might open for new treatment strategies to combat the development of resistance to benzimidazoles. Furthermore, several antibacterial substances have been shown to be substrates for BCRP e.g. ciprofloxacin, enrofloxacin, nitrofurantoin. The development of resistance to antimicrobials is also an increasing threat. One mechanism for development of antimicrobial resistance is that efflux transporters protect the microorganisms against accumulation of drugs. It would be interesting to study the effect of oleic acid on BCRP-mediated efflux of e.g. ciprofloxacin in bacteria.

Finally, many food carcinogens are substrates for BCRP, which means that the absorption of them is normally restricted. The effect of oleic acid on the absorption of such substances, e.g. aflatoxin, in mice would be interesting to study. It could also be investigated if this increased absorption results in a higher mutagenic frequency in for instance the sensitive micronucleus tests in mice.

8 Populärvetenskaplig sammanfattning

Denna avhandling handlar om fetters påverkan på tarmens förmåga att stoppa skadliga ämnen i mat att ta sig in till blodcirkulationen. Fett är livsnödvändigt för alla levande organismer inklusive människa. Det är ett av de viktigaste näringsämnena och behövs för en normal utveckling. Av den energi vi får i oss utgörs 30 – 40 % av fett. Mer än 90 % av fettet som vi får i oss från maten består av triglycerider som bryts ner till fria fettsyror och monoglycerider i mag-tarmkanalen. Fettsyrorna har en lipofil del (fettlöslig) bestående av en kolkedja av olika längd, och en hydrofil del (vattenlöslig) vilket ger dem ytaktiva egenskaper. Den vanligaste enkelomättade (en dubbelbindning i kolkedjan) fettsyran i maten är oljesyra (C18:1, 18 kol och 1 dubbel-bindning). Oljesyra förekommer rikligt i olivolja (55 – 83 %) och i fisk, avokado, nötter och flera andra vegetabiliska oljor. Fleromättade (flera dubbelbindningar i kolkedjan), så kallade omega- 3 fettsyror med kolkedjor som består av 18 eller fler kolatomer, anses vara viktiga för en mängd fysiologiska processer, bland annat inflammations-processer, immunsvar och celltillväxt. Dokosahexaensyra (DHA, C22:6, 22 kol och 6 dubbelbindningar) är en sådan fettsyra, som inte kan produceras i människans kropp och som därför är essentiell, d.v.s. är nödvändig att få i sig genom maten. DHA finns främst i fisk och skaldjur, men även i mjölk, ägg och lever, och säljs även som kosttillskott i form av kapslar.

Det har länge varit känt inom läkemedelsforskningen att man kan öka absorptionen av läkemedel, som normalt absorberas dåligt i tarmen då de tas via munnen, genom att dosera dem tillsammans med ytaktiva ämnen. Dessutom har fettrika måltider visats öka absorptionen av vissa läkemedel som normalt absorberas dåligt.

Det centrala i vår hypotes är att ytaktiva fettsyror som normalt finns i maten, oljesyra och DHA, har egenskaper som gör att de kan öka absorptionen av toxiska ämnen via maten.

Tunntarmen är den plats där de flesta substanser absorberas för att komma in i blodcirkulationen. För att komma dit måste ämnet intas via munnen och därefter transporteras via matstrupen till magsäcken och sen vidare ner i tarmen. När substansen kommit till tunntarmen måste den passera flera barriärer innan den kan tas upp i blodet. I detta arbete har främst två absorptionsvägar undersökts.

Först studerade vi den paracellulära absorptionen, vilken sker mellan tarm-cellerna i det cell-lager som täcker tarmväggen, genom en barriär som kallas tight junctions (TJ). TJ är mycket täta och endast små vattenlösliga molekyler kan passera. Mannitol, som är en sockeralkohol, används ofta som modellsubstans då den absorberas uteslutande genom TJs.

Därefter studerade vi den transcellulära absorptionen. Substansen absorberas då genom tarm-cellerna med hjälp av transport-proteiner. Transport-proteiner kan antingen hjälpa substanser in i cellen och sedan ut i blodomloppet eller så kan de kasta ut substanser från cellen till tarminnehållet genom s.k. efflux. Många substanser som är intressanta ur ett livsmedelstoxikologiskt perspektiv hindras till viss del att komma in i blodet från tarminnehållet genom att de är s.k. substrat för efflux-proteinet BCRP/ABCG2 (breast cancer resistance protein). Exempel på sådana ämnen är stekytemutagenen PhIP, plastingrediensen bisfenol A, mykotoxinerna aflatoxin och ochratoxin, samt vissa läkemedel som används till livsmedelsproducerande djur och som kan ge upphov till resthalter i mat, t ex ivermectin, triclabendazol, albendazole och enrofloxacin. BCRP fungerar då som en barriär genom att kasta ut substanserna, tillbaka till tarminnehållet. Mitoxantron är ett läkemedel som används för behandling av bröstcancer och som ofta används som modellsubstans då man vill studera hur BCRP fungerar. BCRP finns i organ som är viktiga för absorption (tarmepitelet i tunntarmen), utsöndring (lever och njure) och fördelning (blod-hjärn-barriären, placenta barriären).

I detta arbete har försök utförts, *in vitro*, genom att använda tarmepitelceller s.k. Caco-2 celler som ursprungligen kommer från en människa med colon-kancer. Då man odlar Caco-2 celler på filter som sätts i cellodlingsplattor, under ca 21 dagar, bildar cellerna ett tätt monolager av epitelceller med TJs och transportproteiner. Detta monolager av celler kan jämföras med ett tunntarmsepitel.

I första delen av detta arbete var syftet att undersöka om oljesyra och DHA påverkar absorption av, de i livsmedel vanliga kontaminanterna, aluminium och kadmium via TJs i Caco-2 celler. Mannitol användes som modellsubstans för paracellulär absorption. Då cellerna exponerades för DHA samtidigt med aluminium, kadmium eller mannitol så ökade absorptionen av substanserna från den apikala (mot tarminnehållet) till den basolaterala (mot blodet) sidan av

Caco-2 cellmonolagret i förhållande till om cellerna exponerades för substanserna utan DHA. Oljesyra påverkade bara absorptionen av aluminium och mannitol men inte kadmium. Detta innebär att den paracellulära absorptionen av dessa ämnen som normalt absorberas dåligt, ökar vid närvaro av fettsyrorna DHA och oljesyra. Elektronmikroskopiska bilder visar att tarmepitelet och även TJs påverkades negativt av DHA och oljesyra. Cell- cell-kontakterna blev mer diffusa och inte så täta.

I den andra delen av arbetet analyserades om oljesyra påverkar absorptionen av BCRP-substratet mitoxantron både i Caco-2 cell monolager och i mus. Oljesyra ökade absorptionen av mitoxantron från den apikala till den basolaterala sidan av Caco-2 cells-lagret. I mus ökade halten av mitoxantron i blod (serum) men också i tarm, lever, njure och hjärna då mössen gavs mitoxantron tillsammans med oljesyra. I mössen var oljesyrans effekt på absorptionen av mitoxantron tidsberoende vilket tyder på att absorptionen var beroende av hur lång tid oljesyran och mitoxantron låg kvar i tarmen. I cellförsöken undersöktes också om oljesyra påverkar genuttrycket av BCRP. Genuttrycket i Caco-2 celler ökade efter sex timmars exponering för oljesyra men mängden BCRP protein ökade inte. Något ökat genuttryck i tarm från mus kunde inte uppmätas troligen beroende på att exponeringstiden i möss var kortare än för cellerna.

Våra resultat visar att fettsyror som normalt förekommer i mat ökar absorptionen av olika substanser över tarmen, vilken normalt hindrar substanserna att komma in i kroppen. Fettsyrorna verkade via två mekanismer, dels genom att vidga TJs och dels genom att hämma efflux-proteinet BCRP.

Vid riskvärdering av kontaminanter i maten bör man ta hänsyn till att fettsyror i maten kan orsaka att ett ämne absorberas i högre grad än vad man förutsett i djurförsök. Laboratoriedjur äter mat med mindre fett än vad människor gör. Riskbaserade intagsgränser för toxiska ämnen i mat kan vara underskattade då de tagits fram med utgångspunkt från försök på laboratoriedjur, som har en diet med lågt fettintag.

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