Dietary Modulation of Uptake Transporters

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The candidate confirms that the work submitted is his/her own and that appropriate credit has been given where reference has been made to the work of others.

“If you can keep your head when all about you
Are losing theirs and blaming it on you,
If you can trust yourself when all men doubt you,
But make allowance for their doubting too;
If you can wait and not be tired by waiting,
Or being lied about, don't deal in lies,
Or being hated, don't give way to hating,
And yet don't look too good, nor talk too wise:
[…] Yours is the Earth and everything that's in it,
And - which is more - you'll be a Man, my son!”

Rudyard Kipling

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ABSTRACT

Transporters play a determinant role in creating and maintaining physiological balance within the cells. Though, not much information exists on the modulation of transporters, especially in terms of polyphenols and other dietary components.

Initially, a comprehensive database was created, using the high-performance search engine Genevestigator. The database summarises the existing knowledge on selected transporters (OAT1, OAT3, OATP1A2, OATP1B1, OATP1B3, OATP4C1, MRP2, MRP3, BCRP, MCT1, MCT7 and SMCT1). The anatomical distribution of the latters was investigated in the human heart, kidney, liver and intestine. Transcriptional modulation was also assessed, in response to biological mediators, disease, chemicals and drugs. It was shown that while some transporters were modulated from a large number of conditions, others only responded to few. Interestingly, not many dietary compounds were tested, highlighting the limited knowledge existing in this area.

Subsequently, expression of a transporter of interest, the organic anion transporter 3 (OAT3), was assessed in liver HepG2 cells. It was predicted, on the basis of the Ct value, that OAT3 was expressed in the cell line at low levels. Modulation of OAT3, in response to stressors (hydrogen peroxide, *tert*-butyl hydroperoxide and ethanol) at various concentrations and for different time lengths was assessed. It was shown that none of the stressors affected the transporter.

In the same cell line, uptake of the metabolite kaempferol-3-*O*-glucuronide was assessed, to establish whether uptake occurred in a carrier-mediated manner or through passive diffusion mainly. Uptake resulted to be carrier-mediated, although the low Vmax of the transport, close to detection limit, did not make possible further studies to identify the transporter(s) involved in its uptake.

Finally, intestine Caco-2 cells were used to assess modulation of the serotonin transporter from green tea and coffee. For the first time, it was reported that green tea and coffee acted as modulators of serotonin uptake. Whole extracts showed to act in a concentration-dependent way. Physiological concentrations of individual green tea components showed not to have a significant effect on the uptake, however significant effect was observed when using supplement concentrations (equivalent to 7 cups). Physiological concentrations of several coffee components showed to modulate serotonin uptake. Among them, ferulic acid and 5-feruloylquinic acid showed to act in a competitive manner.

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Abbreviations and Units

[^{3}H]-5-HT……...Tritium-labeled serotonin
<……...…..…….More than
>……...……..……Less than
µL…… Microlitre
µM……... Micromolar
5'-CF……....5'-Carboxyfluorescein
5-HT……..……..…….Serotonin
5-hydroxytryptamine…….……. Serotonin
A431…..Human epithelial carcinoma cell line
A549…….Adenocarcinomic human alveolar basal epithelium cells
ABC….....ATP-binding cassette protein
ARC…….Zinc oxide
ASPC-1.....Metastatic pancreas cancer cells
ATF…..... Activating transcription factor 1
ATP……..……..… Adenosine triphosphate
BCRP…… Breast cancer resistance protein
BMP-2…….....Bone morphogenetic protein 2
BPDE…….B[a] P diol epoxide
BSA……...….. Bovine serum albumin
CA....…… Caffeic acid
Ca_2Cl…… Calcium chloride
Caco-2…..……... Colorectal adenocarcinoma
cDNA……...….Complementary DNA
cm……...……Centimetre
COPD……...Cigarette-induced chronic obstructive pulmonary disease
CP-A hTERT…..Barrett's esophagus cell line
CPM……. Count of emission per minute
CQA..……Caffeoylquinic acid
CRE…….. cAMP response element
CREB-1… cAMP response element binding 1
Ct…… Threshold cycle
Ctr…… Control
DAT…….. Dopamine transporter
DMCIN……... Dimethoxycinnamic acid
DMEM... Dulbecco's modified Eagle medium
DMSO…… Dimethyl sulphoxide
DNA…… Deoxyribonucleic acid
DPM…….. Degradation per minute
DTT.....Dithiotreitol
DU145 cells…… Human prostate cancer cells
EC……. Epicatechin
ECG…….. Epicatechin gallate
EGC…….. Epigallocatechin
EGCG…… Epigallocatechin gallate
EtOH……... Ethanol
FA……. Ferulic acid
FBS……..... Fetal bovine serum
FQA…… Feruloylquinic cid
FRET……...Fluorescence resonance energy transfer
g……..Gram
G-CSF……...Granulocyte colony- stimulating factor
Glut……...Glucose transporter
GM-CSF……...Granulocyte macrophage colony-stimulating factor
H_2O_2…….. Hydrogen peroxide
HBSS…… Hank's balanced salt solution
HCl…… Hydrogen chloride
HEK 293.....human embryonic kidney 293 cells
HepG2…… Hepatocellular carcinoma
HNF…….. Human nuclear factor
HOb514……..Human osteoblast cell line
HPBM……Human peripheral blood mononuclear cells
HPLC……..... High performance liquid chromatography
Hs 294 T……Melanoma-derived cell line 294
HUVEC……Human umbilical vein endothelial vein cells
IC_{50}…… Half maximal inhibitory concentration
ICD…….. Intracellular domain
IFN-g.....Interferon gamma
IL…… Interleukin
K-3-*O*-g…… Kaempferol-3-*O*-glucuronide
Kg……. Kilogram
KGF.....Keratinocyte growth factor
Ki……... Inhibition constant
Km……... Michaelis-Menten constant
L……Litre
LC-MS…….. Liquid chromatography mass spectrometry
LDH…… Lactate dehydrogenase
m.....Metre
MAO……..Monamine oxidase
MAT…….. Monoamine transporter
MCT…….. Monocarboxylate transporter
MDA Mb231……..….Mammary gland cells derived from metastatic site
MeG……... Methylglabridin

MEM……Minimum essential medium
mg……... Milligram
Min…….. Minute
mL……. Millilitre
mM……. Millimolar
MONO-MAC-6……...Monocytic cell line 6
MPP^+…… 1-methyl-4-phenylpyridinium
mRNA……... Messenger RNA
MRP……... Multidrug resistance protein
MTT…. Methylthiazolyldiphenyl-tetrazolium bromide
NADP….. Nicotinamide adenine dinucleotide phosphate
NaOH…….. Sodium hydroxide
NBD…… Nucleotide-binding domain
NET……...Norepinephrine transporter
nM…….. Nanomolar
NO…… Nitric oxide
NPrEC……Normal prostate epithelial cells
OAT…….Organic anion transporter
OATP…….....Organic anion transporting polypeptide
OCT……....Organic cation transporter
OCTN…….Organic carnitine transporter
OPM1……Peripheral blood cells from patient with melanoma
OSA…….Oxidant scavenging activity
PAH…….P-aminohippurate
PGE2……Prostaglandin E2
PKC……...Protein kinase C
PMA……....Phorbol myristate acetate
Pmol…….Picomolar
RNA……...Ribonucleic acid
RT……...Room temperature
RT-PCR…….Reverse transcription-Polymerase chain reaction
SDF…….Stromal cell-derived factor
SERT…….Serotonin transporter
shRNA……...Short hairpin RNA
SLC……..Solute carrier transporter
SMCT……Sodium-coupled monocarboxylate transporter
St Dev……..Standard deviation
SW480……...Dukes' type B, colorectal adenocarcinoma cells
tBOOH……... *Tert*-Butyl hydroperoxide
TGF……. Transforming growth factor
THP-1…… Leukemic monocytes
TMD……..Transmembrane domain
TNF……..Tumor necrosis factor
TSA……...Trichostatin A
U251…… Astroglioma cells
U937…….Human leukemic monocyte lymphoma cells
U938……..Monocyte model cells
VAD……..Ventricular assist device
Vmax…….Maximum uptake rate
ZnOAC2……Zinc acetate
Ω…….. Ohm

Chapter 1

Introduction

1.1. Polyphenols

1.1.1. Chemical features

Polyphenols are a group of secondary metabolites, abundantly produced in plants, where they probably act as defence against pathogens and disease (Visioli and Galli, 1997). More than 8,000 polyphenols are currently known (Harborne and Williams, 2000). They are generally small molecules with molecular weights between 200 and 800 Da, structurally characterized by one or more aromatic phenol rings (C6-OH). Though their chemical structure varies considerably from one compound to another (Williamson and Manach, 2005). Differences in the chemical structure determine the unique biological properties of each class and, among the classes, of each compound (Brisdelli *et al.*, 2009).

Based on their chemical structure, polyphenols can be divided into four classes: flavonoids, phenolic acids, lignans and stilbenes (Reboul *et al.*, 2007b) (fig 1-1). Flavonoids can be divided into flavonones, proanthocyanidins, anthocyanins, isoflavones, flavones and flavonols. Phenolic acids include hydroxybenzoic acids and hydroxycinnamic acids. In the human diet, flavonoids are the most abundant and stilbenes the least abundant (Manach *et al.*, 2004).

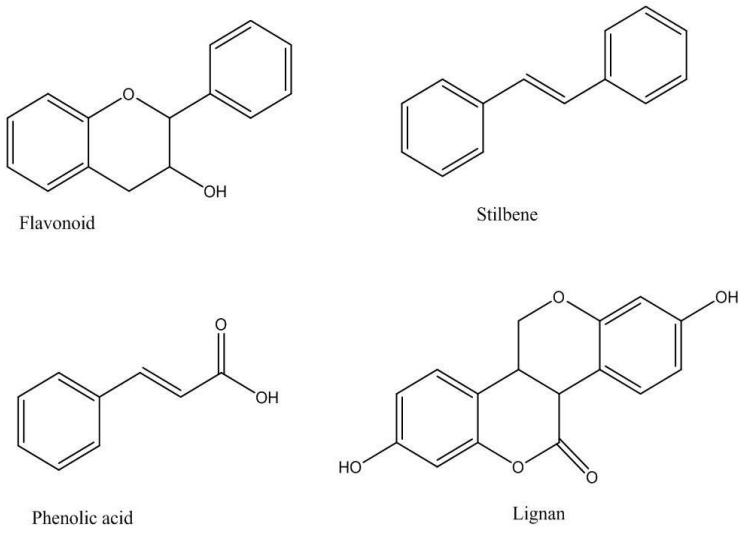


Figure 1-1. Chemical structure of the main polyphenol classes. Polyphenols can be divided into four classes: flavonoids, stilbenes, phenolic acids and lignans.

1.1.2. Dietary sources

Each class of polyphenols includes a variety of members widely distributed in commonly consumed food and beverages.

The dominant source of polyphenols in our diet is represented by beverages. Worldwide tea is the second most consumed beverage, after water, and is rich in polyphenols. Epidemiological studies show that 85 % of the world population regularly drinks tea (Beresniak *et al.*, 2012). Coffee is another highly consumed beverage and it is also rich in polyphenols. Studies show that 80 % of the world population drinks coffee on a daily basis (van Dam, 2008). Another abundant source of polyphenols is fruit, while vegetables contain relatively little amount of these compounds (Graham, 1992).

To give a few quantitative data: one litre of orange juice contains 125-250 mg of the flavonone hesperidin, a litre of grapefruit juice contains 145-638 mg of the flavonoid naringenin, and a cup of coffee contains about 150 mg of chlorogenic acid (Scalbert and

Williamson, 2000). Overall, 1 g/d has been evaluated to be the average intake of polyphenols (Beecher, 2003). The relative amount of each polyphenol ingested largely depends on consumer preferences and on the alimentary habits linked to the culture. For example in some Eastern countries, such as China, a major source of polyphenols is represented by soya and the products derived from it. At the contrary soya is barely present or not present at all in Western diets (Adlercreutz *et al.*, 1991).

It must also be considered that the initial abundance of polyphenols in food does not necessarily correspond to a high intake, due to food processing. For example, apples contain high amount of the flavonol quercetin (1 mg/g of fresh fruit) in the peel, but no quercetin at all nor other flavonols in the pulp. Thus, apple peeling eliminates the total flavonol content (Lee and Mitchell, 2012).

A further important factor to be considered when evaluating the actual polyphenol amount usable within the human cell is the bioavailability. The bioavailability is the amount of compound present in the systemic circulation after oral consumption (Hof *et al.*, 1998).

Polyphenol bioavailability is quite limited. In fact, a significant part of the ingested amount is eliminated from the body. It has been demonstrated that after consumption of 10 to 100 mg of a single compound, levels of that polyphenol in blood rarely rise above 1 μM, despite their local concentrations are certainly much higher than that (Manach *et al.*, 2005). The bioavailability mainly depends on the chemical structure of each polyphenol, which determines the type and amount of metabolites formed and if they will predominantly be absorbed or excreted.

1.1.3. Role in human health

The human body is constantly exposed to oxidative stress, from both endogenous and exogenous sources. The endogenous sources come from our life based on oxygen

consumption, which produces the energy necessary for life but also waste products such as free radicals (Valko *et al.*, 2006). In the long-term free radicals can damage the cell (Tekiner-Gulbas *et al.*, 2013). The exogenous sources include the daily exposure to UV light and pollutants such as smoke (Carnevali *et al.*, 2003).

Oxidative stress is linked to a wide range of degenerative pathologies such as cancer, osteoporosis and cardiovascular disease (Simonian and Coyle, 1996).

Since the endogenous defences of the human body alone are not enough in fighting free radicals, it is essential to consume antioxidants through the diet. The main source of antioxidants in the human diet is represented by polyphenols. They can play a determinant role against oxidative stress, as it has been proved that some of them can help reduce the incidence of a variety of degenerative diseases (Middleton *et al.*, 2000, Ding *et al.*, 2013).

Increasing evidence, showing that polyphenols act as antioxidants, come both from *in vitro* and *in vivo* studies. As polyphenols are reducing agents, *in vitro* they give hydrogen atoms that can neutralise free radicals and protect the biological membranes. *In vivo*, they have been proven to act as regulatory factors of transcription and as antioxidants. As regulatory factors of transcriptions they are connected to many important processes such as cell growth and apoptosis, while as antioxidants they are associated with reducing the risk of chronic and neurodegenerative disease (Nakatani and Kikuzaki, 1995, Williamson and Carughi, 2010).

1.2. Metabolism

The main metabolising organs present in the human body are the stomach, the intestine, the liver and the kidneys (fig. 1-2).

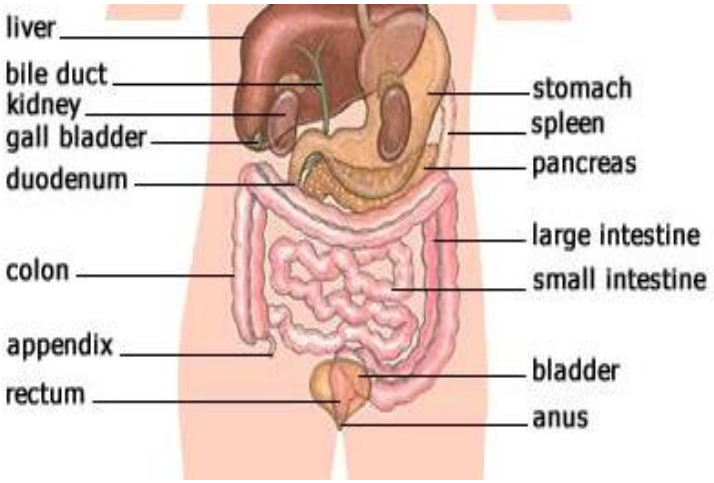


Figure 1-2. Metabolising organs of the human body. The main sites of metabolism in humans are the stomach, the intestine, the liver and the kidneys. All compounds move through these organs to be chemically transformed and finally either retained or excreted.

The metabolic reactions occurring after polyphenol ingestion are critical for the final bioavailability. The metabolism of xenobiotic compounds consists of two groups of reactions, phase I and phase II. Phase I reactions are catalyzed by enzymes such as monooxygenases and P450 (Berdikova Bohne *et al.*, 2007). Phase I metabolism results in the introduction of functional groups, such as carboxylic and hydroxyl groups. Phase II enzymes target the groups introduced during phase I metabolism, triggering in their conjugation (Wong *et al.*, 2011c).

Generally phase I enzymes do not metabolize polyphenols, which are directly conjugated by phase II enzymes. Although the organs and the reactions involved are the same for every polyphenol (fig. 1-3), the outcome strongly depends on the initial structure of the ingested compound.

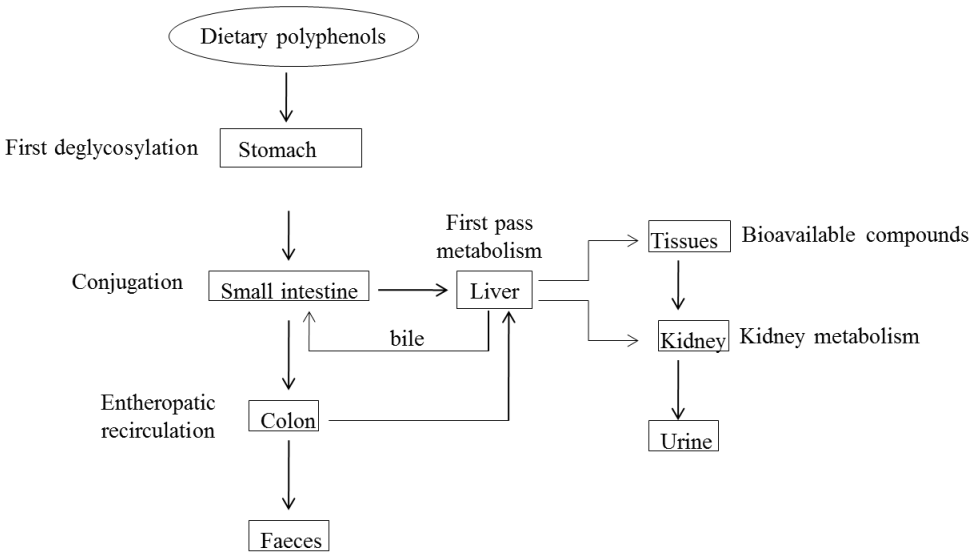


Figure 1-3. Steps of polyphenol metabolism. Polyphenol ingestion is followed by chemical transformation through four steps: deglycosylation, conjugation, first pass metabolism and entheropatic recirculation. These steps are carried out in the main metabolising organs and finally result in absorption or excretion of polyphenol metabolites.

1.2.1. Deglycosylation

The first step of polyphenol metabolism is the deglycosylation in the gut (Setchell *et al.*, 2001). Polyphenols predominantly exist in the sugar-conjugated form. The sugar is either rhamnose or glucose, but different other sugars can occasionally be found, such as arabinose. The number of sugar residues varies from one to three with one being the most recurrent number. Also important are the position of the glycosidic group and if the latter is or is not further substituted (Liu and Hu, 2007). The presence of glycosidic groups on the phenolic compound is critical in inhibiting passage across membranes, because glycosides reduce the compound hydrophobicity. Thus initial enzymatic removal of the glycoside group is required, for the resulting compound to freely diffuse across membranes.

Deglycosilation results in the formation of a polyphenol form, known as aglycone (Manevski *et al.*, 2012).

Different enzymes are involved in the removal of specific sugars. For example the α-rhamnosidases have activity in cleaving rhamnose residues. Some glycosides are removed in the small intestine from endogenously secreted enzymes. Other glycosides are removed later in the colon, from enzymes of the intestinal microflora, such as the α-rhamnosidases (Avila *et al.*, 2009).

1.2.2. Conjugation

Aglycones are rarely found free in blood. Immediately after deglycosylation they are conjugated through methylation, sulfation, glucuronidation or a combination of the three (Liu and Hu, 2002). The hydroxyl groups of the polyphenols are the major target of conjugation, resulting mainly in sulfates and sulfo-glucuronides. Glucuronides are a minor fraction. The methylated form is the least recurrent (Nardini *et al.*, 2009).

The amount of polyphenol ingested is critical in determining the conjugation site. When moderate doses of polyphenols are ingested the conjugation process happens in the intestinal mucosa. If exceptionally high doses of polyphenols are ingested, the main organ involved is the liver. This is due to saturation of the conjugating mechanisms in the intestine (Scalbert *et al.*, 2002). Conjugates physiologically differ from their aglycone form: glucuronidation and sulfation enhance hydrophilicity, whereas methylation enhances lipid solubility (Whitley *et al.*, 2005). Conjugates have low propensity for free diffusion through biological membranes, thus they need active transport. At the contrary aglycones move mainly through passive diffusion.

1.2.3. First-pass metabolism

The liver plays a crucial role in protecting the human body against xenobiotics. Once polyphenols have been conjugated in the intestine they are carried into the liver through the vein porta, where they undergo first-pass metabolism (Scheepens *et al.*, 2010). First pass metabolism consists of further modifications (e.g. sulfation). Subsequently the destiny of the resulting metabolites can be either distribution to body tissues, export through the blood stream to the kidneys or export through the bile to the gut lumen. In the kidneys polyphenols are further modified, consequently they are excreted in the urine. In the gut lumen polyphenols are either excreted in the faeces or further metabolised by the intestinal microflora (Thill *et al.*, 2012).

1.2.4. Entheropatic recirculation

The human colon contains about 10^{12} microorganisms/cm^3. It is estimated that the number of bacteria inhabiting the human gut is ten times greater than the total number of cells in the body (Bjorksten *et al.*, 2001). The gut microflora is essential in digesting un-utilized energy-containing substrates produced in the liver (Laparra and Sanz, 2010).

1.3. Transporters

A determinant role in each of the metabolic steps described above is played by efflux and uptake transporters. Transporters are cellular components inserted into either the apical or the basolateral side of the epithelium of each organ (Keppler, 2004). They can move compounds in or out of the cell. They are called uptake transporters if they move compounds from the extracellular space into the cell cytoplasm. At the contrary efflux

transporters move compounds out of the cells into either the lumen of the organ or the blood stream (fig. 1-4). Efflux transporters were characterized first, whereas uptake transporters have seen the focus of relatively recent interest and a lower amount of information is available for the latter (Rodrigues *et al.*, 2009).

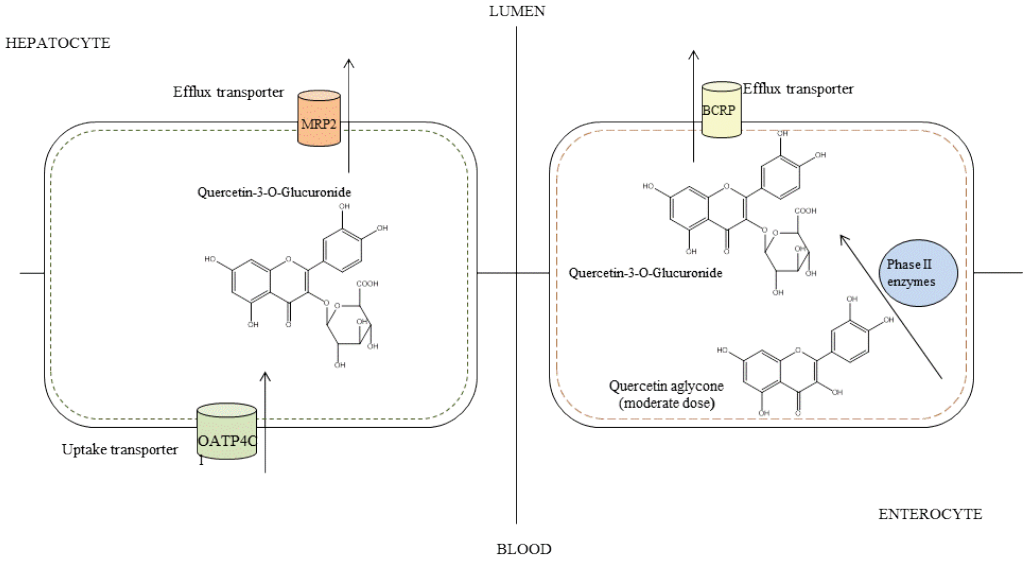


Figure 1-4. Examples of uptake and efflux transport in liver and intestinal cells. Transporters can be divided into uptake and efflux, according to whether they mediate movement from the extracellular space towards the cytoplasm of the cells or in the opposite direction.

Transporters determine whether a particular compound will predominantly be available for the body tissues or be eliminated. For this reason, study of the biochemistry of transporters is a crucial interest. It is known that transporters can be modulated by a wide range of compounds, including dietary molecules, so that our diet is a determining factor for the final balance of absorption/excretion (Katragadda *et al.*, 2006). It is essential to identify which molecules are responsible for transporter modulation and through what mechanism(s).

There are two characterized types of transporter modulation, functional and expressional (fig. 1-5).

Functional modulation applies mainly to efflux transporters and consists of a direct action of the modulator on the transporter. This triggers a conformational change that results in a modification of the amount of compound transported (Higashikuni *et al.*, 2010).

Expressional modulation consists of a change in the amount of mRNA transcribed for a certain transporter, due to the interaction of modulating compounds with the promoter of the gene of interest (Prasansuklab *et al.*, 2011). Either a modulator interacts directly with the gene promoter, acting as a transcriptional factor, or it affects transcription indirectly, by triggering the production of secondary mediators interact with the gene promoter (Wetzler *et al.*, 2000).

It is worth mentioning that although a particular molecule may affect the amount of mRNA produced, this does not always represents a direct evaluation of the corresponding protein levels, which may be modulated by the action of other factors (Zembruski *et al.*, 2011).

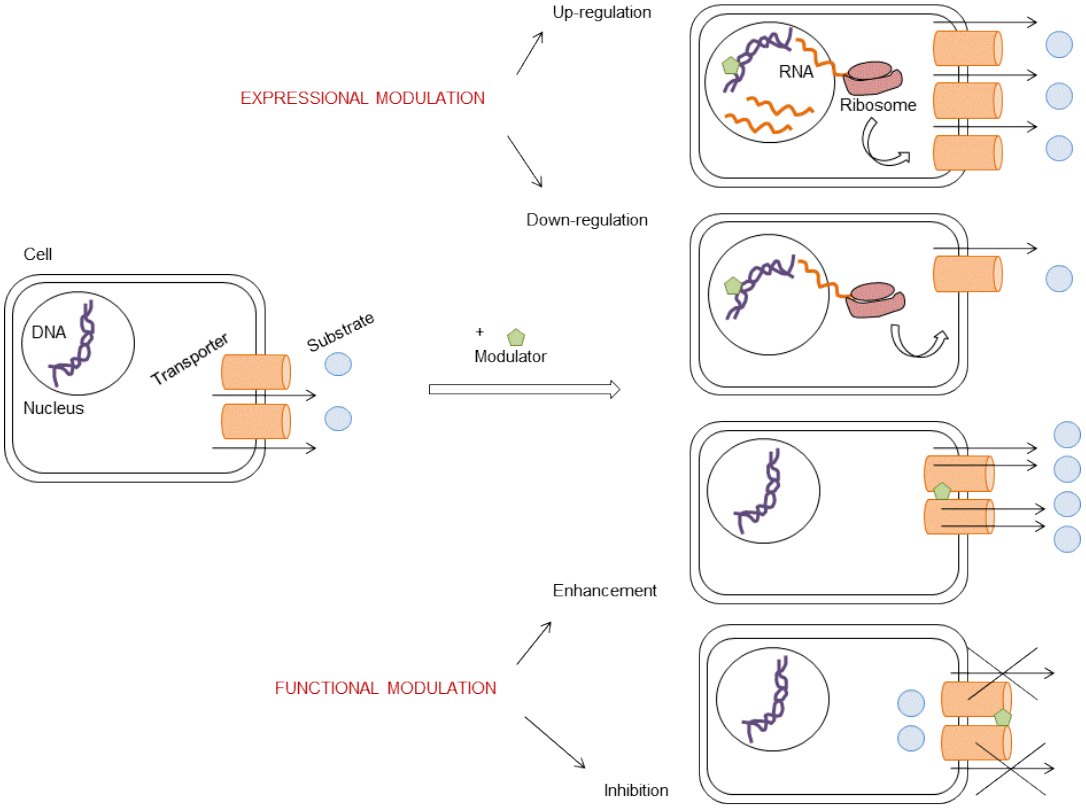


Figure 1-5. Types of transporter modulation: functional and expressional. Functional modulation consists in the direct interaction of modulator with the transporter, resulting in a conformational change. Expressional modulation consists in the interaction of modulators with the gene promoter of the transporter, resulting into a change in the amount of the mRNA transcribed.

Much evidence exists on support of expressional modulation of efflux transporters (Liu *et al.*, 1999, Lespine *et al.*, 2008, Gholami *et al.*, 2014), whereas very few data have yet been provided on support of the expressional modulation of uptake transporters.

The first polyphenols described as capable of interacting with uptake transporters were coffee and cocoa components, with OATP4C1 and OAT3 respectively uptaking sulfate and glucuronide conjugates (Wong et al., 2011b).

1.3.1. Main classes of transporters

1.3.1.1. Efflux transporters

1.3.1.1.1. ATP-binding cassette proteins (ABCs)

The major family of efflux transporters is represented by ABC proteins, which directly couple the transport of compounds to the hydrolysis of ATP. The ABC is the largest family of transmembrane proteins, with members expressed in every existing phyla and members specific to certain phyla. Members excreting and uptaking exist in prokaryotes. Eukaryotes only express members excreting compounds and certain members which do not uptake or excrete but have a role in gene expression modulation and DNA repair (Borst *et al.*, 2006).

The typical structure of an ABC protein is characterized by the presence of two domains, a transmembrane domain (TMD) and a nucleotide-binding domain (NBD). The TMD faces the lumen and consists of α-helices inserted into the cell membrane.

The chemical sequence of the TMD varies greatly according to the substrates transported and it undergoes conformational change upon recognizing the substrate and in order to transport it. The NBD in contrast faces the cytoplasm of the cell and has a conserved sequence, containing the site for ATP binding. Additionally, some ABCs have an intracellular domain (ICD) between the TMD and the NBD (Zelcer *et al*., 2003, Napier *et al.*, 2005).

In the ABCs, the number of TMDs and NBDs varies, with a minimum of two of each domain. At gene level it is also not uncommon for an ABC gene to encode for half a transporter and for the two halves to subsequently combine as either heterodimers or homodimers to form a functional protein (Schinkel and Jonker, 2003).

The effluxing ABCs expressed in eukaryotes are divided into 7 sub-families (ABCA, ABCB, ABCC, ABCD, ABCE, ABCF and ABCG) each with prevalent specificity for

certain substrates. Overall the ABCs transport an extremely wide range of chemically different substrates (Jones and George, 2004). Efflux transporters are distributed with uneven abundance across the biological membranes (fig. 1-6).

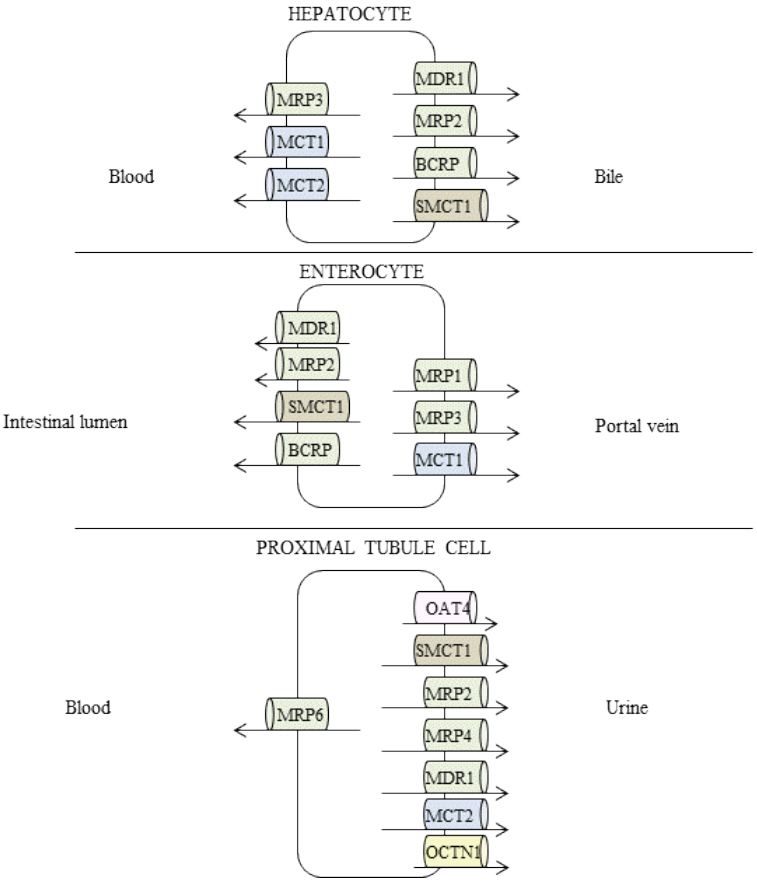


Figure 1-6. Distribution and localization of the main efflux transporters in mammalian liver, intestine and kidney cells (Lubelski *et al.*, 2007, Ganapathy *et al.*, 2008, Pochini *et al.*, 2013, Omlin and Weber, 2013).

1.3.1.2. Uptake transporters

The SLCs (solute carrier transporters) represent the main class of uptake transporters and include several families with their members closely related in terms of substrate specificity

(DeGorter *et al.*, 2012). Distribution of the SLCs varies across the biological membranes of the human organs (fig. 1-7).

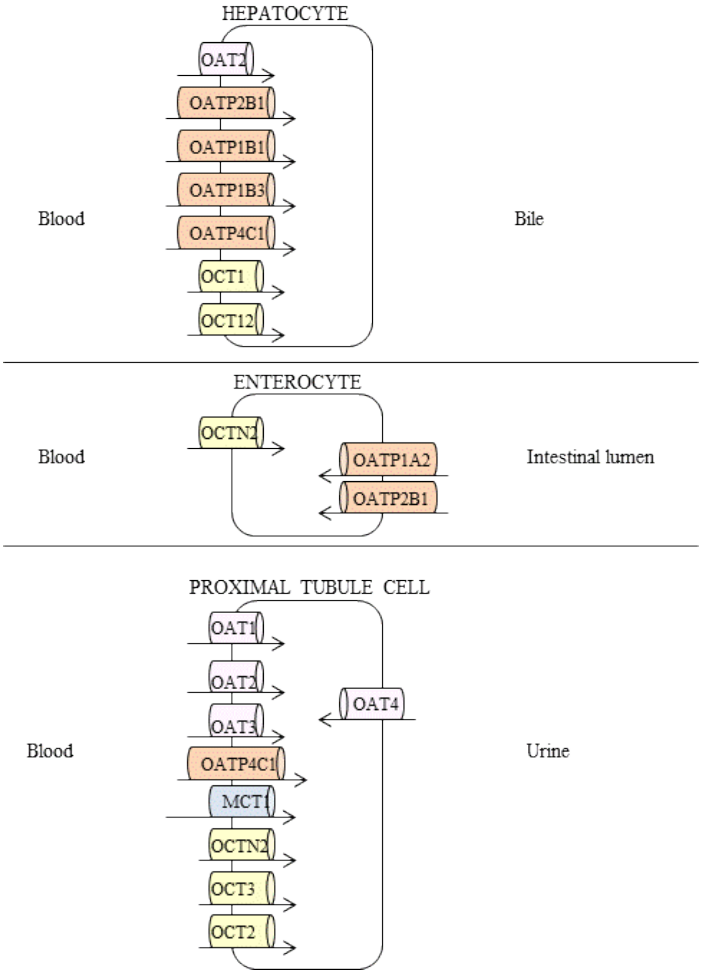


Figure 1-7. Distribution and localization of the main uptake transporters in mammalian liver, intestine and kidney cells (Nigam et al., 2007b, Popovic et al., 2010, Mukherjee et al., 2011).

All of the SLCs play a determinant role in drug and nutrient absorption (Scopelliti *et al.*, 2013). Their understanding is vital for designing effective drugs and functional food and to increase the bioavailability of health-promoting food.

The major families of uptake transporters are OATPs (organic anion transporting polypeptides), OATs (organic anion transporters) and OCTs (organic cation transporters).

1.3.1.2.1. Organic anion transporting polypeptides (OATPs)

The OATPs are characterized by the presence in their structure of twelve transmembrane domains with multiple glycosylation sites. To date 11 members have been identified in humans and divided into six subfamilies: OATP1, OATP2, OATP3, OATP4, OATP5 and OATP6. All are widely distributed. A few members are ubiquitously expressed, while some members are localized in specific organ(s) or tissue(s) (Hagenbuch and Meier, 2004).

It has been reported that OATP1B1, OATP1B3 and OATP2B1 are only expressed in the liver, while OATP4C1 is expressed in the kidney only. Each of them is essential to uptake specific compounds in the organ for further metabolism and final elimination through bile/urine (Yamaguchi *et al.*, 2008, Vasuri *et al.*, 2011).

Literature reports that OATP1A2 and OATP2B1 are expressed in the small intestine, where they contribute to ion absorption (Glaeser *et al.*, 2007). All other OATPs are mainly expressed in peripheral tissues, where they are involved in uptaking hormones (Noe *et al.*, 2007).

OATPs mainly transport type II amphiphilic anions and some cation and non-charged compounds with molecular weights greater than 400 Da (Kullak-Ublick *et al.*, 2001). Although the transport mechanism of OATPs is not yet characterized, it is known that they do not directly use ATP. It is also known that they function in a sodium-dependent manner (Kalliokoski and Niemi, 2009). It is very likely that anionic uptake depends on its exchange with another anion such as glutathione and/or bicarbonate. This hypothesis is consistent with observations that all the OATPs (except for OATP1C1) function at their best at low

extracellular pHs (Nozawa *et al.*, 2004). Conversion of bicarbonate into carbon dioxide and water, triggered by low pH, would determine movement of the bicarbonate ions toward the extracellular space.

1.3.1.2.2. Organic anion transporters (OATs)

The OATs include seven characterized members: OAT1, OAT2, OAT3, OAT4, OAT6, OAT7 and OAT10 (Ahlin *et al.*, 2009). They all display extraordinary broad substrate specificity. OATs transport mainly type I amphiphilic anions, but also some cation and non-charged molecules. In either case the compounds transported are generally small molecules with molecular weights between 400 and 500 Da (Sekine *et al.*, 2000, Burckhardt and Burckhardt, 2003).

OATs transporters are mainly found in the basolateral membrane of the kidney proximal tubules. They work through a sodium-dependent mechanism that relies on two coupled proteins, K^+/Na^+-ATPase and Na^+/dicarboxylate co-transporter 3. K^+/Na^+-ATPase pumps Na^+ out of the cell using ATP. The Na^+ electrochemical gradient is subsequently used from Na^+/dicarboxylate co-transporter 3 to intake α-ketoglutarate. Finally the OATs couple the uptake of organic ions to the exit of α-ketoglutarate. The mechanism of action of OATs gives them the name of active tertiary transporters (Enomoto and Endou, 2005). There are exceptions to the above mechanism. OAT2, for example, does not couple transport to α-ketoglutarate excretion but works in a not yet characterized manner, probably coupling the uptake of compounds to ion efflux (Yohannes *et al.*, 2013).

For the structure of the OATs a model has been proposed, with twelve transmembrane domains and multiple glycosylation sites (Burckhardt and Burckhardt, 2003).

OAT1 and OAT3 are found mainly in the human kidneys and in smaller amount in skeletal muscle, brain and eye. OAT1 has an extraordinarily broad range of substrates, including a wide range of drugs and endogenous compounds, such as some neurotransmitters and prostaglandins (Lopez-Nieto *et al.*, 1997).

OAT3 was firstly identified as a transporter for estrone-3-sulfate but was then discovered to transport many other substrates, such as taurocholate (Nigam et al., 2007a).

OAT2 is mainly expressed in the basolateral membrane of liver and in minor amount in the kidney proximal tubules. OAT2 has a much less broad range of substrates compared to OAT1 and OAT3. Substrates include steroid hormones (Cha *et al.*, 2001).

OAT4 is exclusively expressed in humans, where it is found in the placenta and the apical membrane of the kidney proximal tubules. OAT4 acts both as an uptake and as an efflux transporter towards organic anions. It acts by coupling their movement out of the cell with the entrance of other ions such as chloride (Hagos *et al.*, 2008). The substrate specificity of OAT4 for effluxed compounds is widely overlapping that of OAT1 and OAT3. For this reason it is believed that the three transporters operate in a synchronized manner to effectively eliminate organic compounds through the urine (Ekaratanawong *et al.*, 2004).

1.3.1.2.3. Organic cation transporters (OCTs)

OCTs are mainly expressed in liver, kidney and brain, with varying abundance. OCTs mainly transport cations such as monoamine neurotransmitters, but can also transport some anions and zwitterions (Ciarimboli, 2011). So far about 20 members have been discovered but for the majority very little is known (Takane *et al.*, 2008). Localization of OCT1, OCT2, OCT3 and OCT12 has been assessed. OCT1 is expressed in the human liver and brain (Lee *et al.*, 2009). OCT2 is mainly found in the kidneys and in minor amounts in the brain

(Kiefer et al., 1998). The prime sites of OCT3 expression are brain and kidney (Wu *et al.*, 2000). In the kidney OCT3 transports a wide range of solutes including histamine, serotonin, norepinephrine and dopamine (Ciarimboli, 2008, Baganz *et al.*, 2008).

OCT12 is found in the kidney, where deficiency has been linked to hyperuricemia and hypouricemia (Motohashi *et al.*, 2013).

Special importance has to be given to a subclass of OCTs, the OCTNs (organic carnitine transporters).

1.3.1.2.3.1. The organic carnitine transporters (OCTNs)

The OCTNs are responsible for the maintenance of carnitine levels in the body by bidirectional transport (Lahjouji *et al.*, 2001). Since carnitine is crucial in the mitochondrial utilization of long chain fatty acids, maintenance of carnitine balance plays an important role in energy production (Lim *et al.*, 2010).

Four OCTNs have been discovered: OCTN1, OCTN2, OCTN3 and OCTN4, with varying abundance within human organs. OCTN1 is expressed almost ubiquitously, except in adult liver. OCTN1 has relatively low substrate affinity. It is thought to act maximally by secreting carnitine and short-chain esters at the brush border membrane of kidneys (Lamhonwah and Tein, 2006). OCTN2 uptakes carnitine in the majority of human organs (Grube *et al.*, 2004).

In contrast to OCTN1, OCTN2 has high affinity for its substrate and can be quantitatively considered the main carnitine uptaking transporter. OCTN2 is highly expressed in skeletal muscle, heart, placenta and kidney and weakly expressed in lung, liver and brain (Rodriguez *et al.*, 2002). OCTN3 is mainly expressed in the testis, where it uptakes carnitine with higher affinity than OCTN1 and OCTN2. Its activity in other organs is not yet fully

characterized (Januszewicz *et al.*, 2009). Tissue distribution and mode of action has not yet been characterized for OCTN4.

Interestingly it is not yet known how carnitine is secreted from the human liver, although the liver is the main site of carnitine synthesis (Rosenbaum *et al.*, 2013) and thus release from this organ is the focus of numerous studies.

Mechanism of action has only been characterized for two members of the OCTNs, OCTN1 and OCTN2. OCTN1 works in a pH-dependent manner. OCTN2 works both in a sodium-dependent and in a sodium-independent manner (Garrett *et al.*, 2008).

1.3.1.2.4. Monoamine transporters (MATs)

Another important group of transporters are MATs, all indispensable in maintaining the intracellular/extracellular balance of monoamine transmitters (Gerevich *et al.*, 2000). There are three main members of the MATs: serotonin reuptake transporter (SERT), dopamine transporter (DAT) and norepinephrine transporter (NET). SERT, DAT and NET each have a specific substrate, serotonin, dopamine and norepinephrine respectively (Gainetdinov and Caron, 2003).

MATs are located in the perisynaptical space, outside the presynaptic cleft. They re-uptake transmitter molecules back into the cytoplasm of the presynaptic neuron. Their role is crucial for neurotransmitters to be recycled. In fact they terminate one signalling cycle and play a determinant role in the start of a new one (Raap and Van de Kar, 1999).

Certain foods are known to directly increase monoamine neurotransmitter levels, by providing their chemical precursors. Other foods indirectly alter serotonin levels (Kim *et al.*, 2013). A diet rich in milk and eggs, for example, provides tryptophan, precursor of

serotonin. Moreover, a diet rich in carbohydrates increases serotonin release through insulin secretion (Fernstrom and Fernstrom, 1995).

SERT can be found primarily in the central nervous system, in the platelets and in the gastrointestinal tract (Kish *et al.*, 2005). In recent years evidence has highlighted that serotonin is mainly located in the gut (approximately 90 %), where it is synthesized and stored. Synthesis and storage occurs in the enterochromaffin cells, lining the intestinal lumen. It appears that serotonin must play a fundamental role in regulating the gastrointestinal functions (Wheatcroft *et al.*, 2005). In fact, is has been shown that serotonin imbalance is the cause of a range of gastrointestinal dysfunctions, such as irritable bowel syndrome (Wang *et al.*, 2007). Studies have shown that in patients with irritable bowel syndrome the serotonin levels in the gut were much lower than in healthy volunteers (Crowell, 2001). The mechanisms through which serotonin affect gut functions and the pathophysiology of irritable bowel syndrome are not yet fully characterised, although there is evidence that SERT plays a role. SERT removes the serotonin from the post-synaptic space. After serotonin binds to its receptors and it initiates a signalling cascade in the post-synaptic cells, SERT mediate reuptake in the releasing cell. This links to serotonin metabolism and recycle (Jafari *et al.*, 2011). Studies have shown that irritable bowel syndrome patients had increased expression of SERT in the colon, and therefore, increased serotonin reuptake, compared to healthy donors (Coates *et al.*, 2004).

DAT is primarily found in the *substantia nigra* of the human brain (Ciliax *et al.*, 1999).

It has been suggested that DAT plays an important role in maintaining the intracellular/extracellular balance of dopamine in the dendrites of the *substantia nigra* (Liu et *al.*, 2007).

NET expression is restricted to noradrenergic neurons. NET is found in the cell body, dendrites and axons (Sager and Torres, 2011). Both NET and DAT can re-uptake

norepinephrine (Galli *et al.*, 1995). NET is also essential in maintaining the homeostatic balance in the presynaptic neurons (Torres *et al.*, 2003).

In terms of protein structure and mechanism of action, the MATs are 12 transmembrane domain proteins (Field *et al.*, 2010). They act in a sodium-dependent manner, coupled to the Na^+/K^+ pump. Three Na^+ ions bind to the transporter. Subsequently the protonated form of the neurotransmitter (serotonin, dopamine or norepinephrine) and Cl^- ions also bind to the transporter. Binding of chlorine ions is necessary for net transport to happen. The initial binding of Na^+ and Cl^- triggers a conformational change of the transporter, which results in the uptake of the neurotransmitter and ions into the cell cytoplasm. When the neurotransmitter is released and the transporter is unbound, two intracellular K^+ ions bind to it. This links to the transporter regaining the initial conformation, facing the synaptic cleft and ready to undergo the next transport cycle (Kuzelova *et al.*, 2010).

The MATs expressed in the nervous system are key factors in the treatment of mental illness (Min *et al.*, 2009), thus their regulation is the focus of much research. However much less research has been so far published concerning the role of MATs outside the nervous system, where they probably play equally important functions.

1.3.2. Interaction of uptake transporters with polyphenols

Polyphenols interact with uptake transporters in two ways. They either act as substrates or as modulators. It is not uncommon for a compound to be both substrate and modulator of a specific transporter (Rodriguez-Proteau *et al.*, 2006).

1.2.3.1. Polyphenols as substrates of uptake transporters

A few reports exist, identifying polyphenols acting as substrates for uptake transporters.

A study showed that ellagic acid from berries was taken up from OAT4, expressed in oocytes (Whitley *et al.*, 2005).

It was shown that, in cells expressing OAT1, the uptake of the aglycones morin and silybin was 3-fold higher compared to control cells, not expressing OAT1 (Hong *et al.*, 2007).

Interaction of quercetin and genistein conjugates with OAT1 and OAT3 was assessed. Human embryonic kidney cells (293 H) over-expressing OAT1 and OAT3 respectively were used. Genistein-4'-*O*-sulfate and quercetin-3'-*O*-sulfate were found to be substrates of OAT1. Daidzein-7-*O*-glucuronide, genistein-7-*O*-glucuronide, glycitein-7-*O*-glucuronide and quercetin-3'-*O*-glucuronide appeared to be substrates of OAT3 (Wong *et al.*, 2011c).

Quercetin-3'-O-sulfate was found to be substrate for both OAT4 and OATP4C1. Quercetin-3'-sulfate uptake was 2.3- and 1.4-fold higher in cells overexpressing OAT4 and OATP4C1 respectively, than in control cells (Wong *et al.*, 2012).

It was shown that both EGCG (epigallocatechin gallate) and ECG (epicatechin gallate) were substrates of OATP1A2 and OATP1B3, expressed in both enterocytes and hepatocytes (Roth *et al.*, 2011).

1.2.3.2. Polyphenols as modulators of uptake transporters

1.2.3.2.1. Polyphenols as competitive modulators

Ellagic acid was proven to be a potent inhibitor of OAT1 activity, inhibiting uptake with an IC_{50} of 207 nM (Whitley *et al.*, 2005)

Silybin and morin were shown to be competitive inhibitors of OAT1 with IC_{50} values of 25 and 0.5 μM, respectively. Concentrations up to 400 μM of the two aglycones were also tested on OAT3, displaying lower inhibition than on OAT1 (Hong *et al.*, 2007). Consistent with these results, a study from the same group demonstrated that following morin

administration, renal clearance of the anti-cancer drug methotrexate was decreased by 42 %, resulting in improved availability of the drug in the blood stream. Subsequently combined effect of morin and methotrexate was assessed in a cell model. In agreement with the previous findings, co-incubation of cells with morin and methotrexate resulted in inhibition of methotrexate uptake (Hong *et al.*, 2008).

The effect of the natural phenol curcumin was assessed on a wide range of human transporters using human volunteers with cigarette-induced chronic obstructive pulmonary disease (COPD). To date corticosteroids are considered the most effective anti-inflammatory drugs. However people with COPD develop corticosteroid resistance and thus chronic inflammation. Curcumin is well known for its antioxidant, anti-cancer and anti-inflammatory properties (Aggarwal *et al.*, 2013). Patients with COPD were administered curcumin (1 % of the daily diet) for several weeks. After curcumin administration, inflammation markers showed to decrease by 87 % compared to control patients (Moghaddam *et al.*, 2009).

Quercetin-3'-*O*-sulfate was shown to be a potent inhibitor of OAT1 (IC_{50} = 1.22 µM), while quercetin-3'-*O*-glucuronide and quercetin-3-*O*-sulfate inhibited OAT3, with IC_{50} of 0.43 µM and 1.31 µM respectively (Wong *et al.*, 2012).

A study demonstrated that the ginkgo flavonoids kaempferol, apigenin and quercetin were inhibitors of OATP1A2 and OATP2B1 in HEK 293 cells over-expressing the transporters. Quercetin proved to be the most potent inhibitor (Ki on OATP2B1 = 8.7 µM and on OATP1A2 = 22 µM on OATP1A2), followed by kaempferol (Ki on OATP1A2 = 25 µM and on OATP2B1 = 15 µM) and apigenin (Ki on OATP1A2 = 32 µM and on OATP2B1 = 21 µM) (Mandery *et al.*, 2010).

Another research group investigated the effect of naringin and hesperidin from grapefruit juice and orange juice on OATP1A2, using transfected HeLa cells. Competitive inhibition of the anti-histamine drug fexofenadine, taken up through OATP1A2, was observed after

treatment with grapefruit juice or orange juice. IC_{50} values were 3.6 and 2.7 µM respectively (Dresser *et al.*, 2001).

Modulation of OCT1 and OCT3 from red and white wine components was assessed, using 1-methyl-4-phenylpyridinium (MPP^+), a model substrate for OCT1 and OCT3 (Martel *et al.*, 2001).

Caco-2 cells, expressing OCT1 and 3, were used to reveal that alcohol-free red wine increased uptake of MPP^+ in a concentration-dependent manner. Alcohol-free white wine only slightly decreased uptake of MPP^+. In each step of this study, the effect on OCTs was confirmed through co-incubation of wine with OCT inhibitors (Monteiro *et al*., 2005a).

In another study, the effect of individual wine polyphenols (myricitin, catechin, resveratrol and quercetin) was also tested on Caco-2 cells. Myricitin, catechin and quercetin decreased MPP^+ uptake, with quercetin being the strongest inhibitor. Resveratrol displayed no effect (Martel *et al.*, 2010).

The effect of green and black tea on MPP^+ uptake was tested in Caco-2 cells. Both teas showed to increase MPP^+ uptake by several fold and in a concentration-dependent manner. The highest effect came from treatment with green tea. When tea was co-incubated with OCT inhibitors, the effect was attenuated (Monteiro *et al.*, 2005b).

A study assessed the effect of five procyanidin fractions on uptake of MPP^+ in Caco-2 cells. Extracts with increasing structural complexity were produced using grape seeds and used for 60 minutes at a concentration of 600 µg/mL. The work showed that increasing structural complexity related to increased apical uptake of MPP^+ (Faria *et al.*, 2006).

Modulation of MATs from polyphenols has been shown. Apigenin and luteolin, extracted from the fruit *Perilla frutescens*, were tested on wild-type dopaminergic cell lines. These cell lines had high specificity for dopamine, serotonin and norepinephrine uptake. Treatment of the cells with apigenin and luteolin enhanced uptake of the neurotransmitters, with luteolin showing greater potency than apigenin (Zhao *et al.*, 2010).

Polyphenols have been proven to trigger inhibition of serotonin re-uptake. HEK-293 cells were used to stably express SERT and incubated with 50 µ of the following isoflavans from liquorice: glabridin, 4′-*O*-methylglabridin (4′-OMeG), glabrene, resorcinol, 2′-*O*-methylglabridin (2′-OMeG), genistein and daidzein. Glabridin, 4′-OMeG and glabrene inhibited SERT by 60 %, 53 % and 47 % respectively. The other tested compounds proved to have no effect on SERT activity (Ofir *et al.*, 2003).

1.2.3.2.2. Polyphenols as modulators of the mRNA levels

Expressional modulation of uptake transporters is relatively little investigated to date. The first study to show modulation of the mRNA levels of an OAT transporter was carried out using nitric oxide (NO). NO is a small lipid-soluble molecule, already known to play a significant role in modulating gene expression in many cells (Rossig *et al.*, 2000).

RT-PCR showed that NO down-regulated the mRNA levels of rOAT2, expressed in rodent liver. It was also shown both *in vivo* and *in vitro* that compounds inducing NO production down-regulated rOAT2 (Cha *et al.*, 2002). For the *in vivo* experiments, rats were intraperitoneally administered lipopolysaccharide, a bacterial component known to induce NO production (Shultz and Raij, 1992). For the *in vitro* experiments, hepatocytes were treated with spermine NONOate, another inducer of NO production (Yin and Dusting, 1997).

Curcumin was found to modulate OAT1 and OAT3 in human monocytic cells (U937). Cells, expressing OAT1 and OAT3, were treated with the stressing agents hydrogen peroxide or cigarette smoke extracts. Both stressors displayed to down-regulate OAT1 and OAT3. When, after exposure to the stressing agents, cells were treated with curcumin (1 µM) for 18 hours, RT-PCR showed up-regulation of OAT1 and OAT3 mRNA levels. Thus

curcumin showed to revert the effect of the stressing agents and restore the mRNA levels of OAT1 and OAT3 (Meja *et al.*, 2008).

1.4. Aims and objectives

In this work the main aim was to gain a better understanding of the function, localization and modulation of selected transporters.

In order to achieve that, two different approaches were used: one based on the use of bioinformatic resources and another based on the application of classical molecular biology techniques, using cultured cell lines.

The main focus of the present work was to assess the impact of dietary compounds, specifically polyphenols, on the expression of selected human transporter genes. Not much information yet exists on the impact of food compounds on transporter expression, therefore it is necessary to further investigating this field. Understanding the impact of dietary compounds on transporters can lead to identifying dietary products and product components that positively affect human health. This knowledge is critical for designing food supplements and functional food.

Transporters play a crucial role in every biological process, because they determine movement of compounds within cells and organs, thus gaining understanding on the impact that modulatory conditions have on them is essential to design drugs and drug-based therapies.

1.4.1. Creation of a comprehensive database, based on the online application Genevestigator, a high performance search engine for gene expression.

Twelve transporters were initially selected on the base of their known involvement in polyphenol transport. They were also selected on the base of the current research interests of the research group.

A database was created, using the bioinformatic tool Genevestigator, in order to have as much information as possible on the selected transporters. This information could be used as a starting point to further investigate the transporters of interest.

In the database:

1. The recent information about the anatomical distribution of each transporter was extracted.
2. The available information on how the selected transporters behave in response to chemicals, drugs and chemical mediators was summarised.
3. Information on the expressional modulation of transporters from disease (cardiovascular and metabolic diseases and afflictions of the gastrointestinal organs) was collected.

1.4.2. Assessment of functional and/or transcriptional modulation of selected uptake transporters in different cell lines: OAT3 in HepG2 cells and SERT in Caco-2 cells.

Characterisation of kaempferol-3-*O*-glucuronide uptake in HepG2 cells.

Two Individual transporters of interest were selected and exposed to a range of compounds. Transcriptional and/or functional modulation, in response to the selected compounds was assessed and characterized.

- OAT3 was the first target to be selected. Recent studies carried out in the research group have shown that OAT3 takes up polyphenol conjugates in human cell models (Wong et al., 2011a).

  Initially, the mRNA levels of OAT3 (*SLC22A8*) were evaluated in the human liver cell line HepG2. Assessment of transcriptional and functional modulation was subsequently carried out. Three stressors (hydrogen peroxide, t-*butyl*-hydroperoxide and ethanol) were selected. These compounds were selected because *in vivo* they interact with the liver. They are both metabolized in this organ, impacting on its physiology.

  Assessment of the functional modulation was carried out using the model substrate 5'-carboxyfluorescein. Effect of the stressing agents was evaluated by quantifying through HPLC the amount of intracellular 5'-carboxyfluorescein after pre-incubation with various concentrations of each stressor. Modulation of the mRNA levels was evaluated through RT-PCR, after incubation with each stressor.

- Kaempferol is the second most abundant flavonol in the human diet, after quercetin. Little is known about the cellular uptake of kaempferol-3-*O*-metabolites in the

human body. Kaempferol-3-*O*-glucuronide was selected. Liver HepG2 cells were used to assess whether kaempferol-3-*O*-glucuronide predominantly occurred *via* active transport, mediated from a carrier, or passive diffusion. If uptake proved to be carrier-mediated, the final aim would be to identify the transporter(s) involved and to modulate transport using dietary compounds.

- SERT is a transporter known to play a role in appetite modulation. Recent studies have also associated polyphenols with appetite. Of particular interest are green tea and coffee, because high consumption of these beverages has been linked to appetite suppression. Green tea and coffee whole extracts and individual green tea and coffee compounds were tested as functional modulators of SERT in Caco-2 cells.

Chapter 2

Material and Methods

2.1. Equipment and chemicals

2.1.1. Equipment

Below is a list of the equipment used for the experiments (Table 2-1).

Table 2-1. List of equipment used in the experiments. The name of the machine, the experimental use and the manufacturing company are reported.

Equipment	Experiment	Manufacturer
Cecil CE 201 spectrophotometer	Absorbance measurement	Cecil Instruments Ltd. Cambridge, UK
96 well-based Multiskan Ascent plate reader	Absorbance measurement	Thermo Fisher Scientific, Waltham, MA
Agilent 1200 series liquid chromatography system, with fluorescence detector and diode array	HPLC	Santa Clara, CA, USA
Zorbax Eclipse XDB-C_{18} Rapid Resolution HT Column	HPLC	Santa Clara, CA, USA
NanoDrop ND-1000 spectrophotometer	RNA quantification	Agilent technology, UK
Genevac, EZ-2 plus model, was used for sample concentration and stock preparation	Sample concentration and stock preparation	Genevac Ltd. Ipswich, UK
StepOne™ Real-time PCR system	Reverse transcription and real-time PCR	Applied Biosystems, Foster City, CA
Tri-Carb 1600TR scintillation counter	Radioactivity count	Packard Instruments, UK

All the experiments were carried out using ultra-pure water, obtained with a Milli-Q water purifying system (Millipore, Milford, MA, USA).

The Milli-Q system employees a series of successive filtration and deionization steps aimed to obtain ultra-pure water. Water purity is related to water resistivity: the highest the resistivity the lower the amount of charge-carrying ions. Typically water is considered ultra-pure when resistivity is ≥ 18.2 MΩ·cm at 25 °C (Huang *et al.*, 1995).

2.1.2 Chemicals

A list of the chemicals used in the experiments is given below (Table 2-2).

All the general laboratory consumables not shown in the table were purchased from Fisher Scientific (Waltham, MA).

Table 2-2. List of chemicals used in the experiments. For each chemical the origin source is given.

Source	Compound
Sigma-Aldrich (St. Louis, MO)	5'-carboxyfluorescein (5'-CF)
	Methylthiazolyldiphenyl-tetrazolium bromide (MTT)
	Kaempferol-3-*O*-glucuronide (k-3-*O*-g)
	Ferulic acid (FA)
	3,4-dimethoxycinnamic acid (3,4-DMCIN)
	Caffeine
	Imipramine
	Bovine serum albumin (BSA)
	Unlabelled serotonin (5-HT)
	Egallocatechin gallate (EGCG)
	Epigallocatechin (EGC)
	Epicatechin gallate (ECG)
	Epicatechin (EC)
Sigma-Aldrich (St. Louis, MO)	Fetal bovine serum (FBS)
	Dulbecco's Modified Eagle Medium (DMEM)

	amphotericin B penicillin-streptomycin
Roche Applied Science (West Sussex, UK)	Bradford reagent: Coomassie Brilliant Blue G-250 Cytotoxicity detection kit (LDH)
Fisher Scientific (Waltham, MA)	*Tert*-Butyl hydroperoxide (tBOOH) Hydrogen peroxide (H_2O_2) Ethanol (EtOH)
Applied Biosystems (Carlsbad, California)	Primers for OAT3 (SLC22A8, Hs01056647_m1) Primers for GAPDH (4326317E) Primers for SERT (SLC6A4, Hs00984349_m1) RNAqueous® kit RNA-to-cDNA Master Mix PCR Master Mix
Perkin-Elmer (Boston, MA, USA)	Radiolabeled serotonin ([3H]-5-HT), with specific activity of 250 µCi/mmol
Extrasynthese (Genay, France)	3-caffeoylquinic acid (3-CQA) 5-coffeoylquinic acid (5-CQA) Caffeic acid (CA)
Nestlé (Lausanne, Switzerland)	Coffee powder (green blend), commercially available Green tea (soluble powder) 3,4-dicaffeoylquinic acid (3,4-diCQA) 3,5-dicaffeoylquinic acid (3,5diCQA)
Kind gift of Prof. Mike Clifford	5-feruloylquinic acid (5-FQA)
ATCC (Manassas, VA)	Caco-2 (colorectal adenocarcinoma) cells HepG2 (hepatocellular carcinoma) cells
Gentaur (Brussels, Belgium)	Enzyme chlorogenate esterase (CES; *Aspergillus japonicus* EC 3.1.1.42) (≥15 U/g)
National Diagnostics (London, UK)	Ecoscint XR scintillation fluid
Perkin Elmer, Life Analytical Sciences, (Beaconsfield, UK)	[^{3}H]-5-HT (tritium-labelled serotonin) ^{3}H Standard

2.2. Cell line maintenance

2.2.1. Colorectal adenocarcinoma (Caco-2) cells

All the plastics used for cell culture were from Corning (Corning Costar Corp., Cambridge, MA).

Caco-2 (colorectal adenocarcinoma) (HTB-37^{TM}) cells were routinely cultured in 75 cm^2 flasks in humidified 5 % CO_2/O_2 atmosphere, at 37 °C. Dulbecco's Modified Eagle Medium (DMEM) was supplemented with 0.25 μg/mL amphotericin B, 100 U/ml penicillin-streptomycin and 15 % heat inactivated fetal bovine serum (FBS).

For the experiments, cells were seeded in 6 well plates. Seeding density was 0.283 x 10^6 cells/well. Cells were used for the experiments between day 6 and 15 post-confluence. The culturing conditions and the medium used for the experiments were the same as when culturing in flasks, but FBS percentage was reduced to 10 %.

All the experimental work was carried out using cells between passage 38 and 50.

2.2.1.1. Optimisation for serotonin uptake experiments

Optimisation, to determine how many days post-confluence were to be used for serotonin uptake experiments was carried out as follows. Expression of the gene encoding for the serotonin reuptake transporter, *SLC6A4*, was assessed between day 5 (day cells reached confluence) and 21 post-seeding, through quantitative RT-PCR. After cells reached confluence, *SLC6A4* mRNA displayed to increase for 5 days. After 5 days from confluence, the mRNA levels reached a plateau and remained constant until day 16 post-confluence (fig. 2-1). On the base of these observations, all the experiment aiming on SERT were carried out using Caco-2 cells between day 6 and 15 post-confluence.

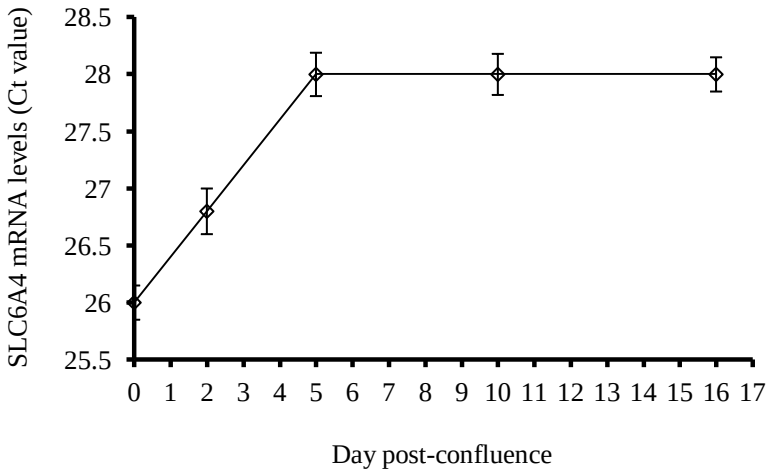


Figure 2-1. Assessment of SERT (*SLC6A4*) expression at different days post-confluence. Caco-2 cells were seeded in 6 well plates and cultured at 37 °C in humidified 5 % CO_2/O_2 atmosphere. Total mRNA was extracted and *SLC6A4* quantified through quantitative RT-PCR for 16 days post-confluence. The results reported are average of triplicates, obtained extracting the mRNA from individual wells, ± St. Dev.

2.2.2. Hepatocellular carcinoma (HepG2) cells

HepG2 (hepatocellular carcinoma) (HB-8065^{TM}) cells were routinely cultured in 75 cm^2 cell culture flasks, under a humidified 5 % CO_2/O_2 atmosphere at 37 °C. The medium, DMEM, was supplemented with 10 % fetal bovine serum and 100 U/ml penicillin. When seeding cells in 12 well plates, seeding density was of 2 x 10^5 cells/well. Experiments were performed with cells between passages 75 and 85 as soon as cells reached confluence, generally 72 h post-seeding.

2.2.3. Assessment of cytotoxicity and cellular viability

Cell viability and cellular integrity were determined using two methods, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and the lactate dehydrogenase (LDH) activity test. These two tests were performed to optimise the concentration of each compound to safely be used on the cells. The assays determine the percentage of cells that are viable and the percentage of cells which have undergone membrane damage, respectively. The MTT and LDH assays can be used as indicators of the cytotoxicity of selected compounds. On the basis of the cytotoxicity assays, all the experiments were carried out using concentrations of the compounds of interest so that cell loss/damage $\leq$ 15 %.

2.2.3.1. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

The MTT test is a colorimetric assay. It is based on the measurement of the activity of cellular oxidoreductase enzymes. These enzymes reduce the MTT reagent to formazan, giving a purple colour. Dye reduction depends on NAD(P)H presence, which is produced in high amount in cellular metabolic reactions. Thus, the more viable cells are present the more MTT reduction is going to occur (Mbanya *et al.*, 2001).

For the MTT test, confluent cells grown in 6 well plates were used. Cells were initially incubated with a range of concentrations of the compound to be tested for cytotoxicity (50-1000 µM for t-BOOH, 50-500 µM for H_2O_2, 0.3-2 mM for EtOH, respectively). The incubation conditions (temperature and time of incubation) were selected to be the same as in the final experiments.

Subsequently, cells were washed once with Hank's balanced salt solution (HBSS) and incubated for 4 h, at 37 °C, with 1 mL fresh medium containing 0.5 mg/mL MTT.

Incubation medium was removed and cells were incubated for 30 min, at room temperature, in the dark and under shaking condition, with 1 mL/well de-staining solution. De-staining solution contained 100 mM HCl and 10 % Triton X-100 in isopropanol. All the de-staining solution was collected after 30 min. 300 µL of it were diluted 1:2 and used for absorbance measurement at 595 nm. A 96 well-based plate reader was used to record absorbance. Fresh de-staining solution was used for the blank (fig. 2-2).

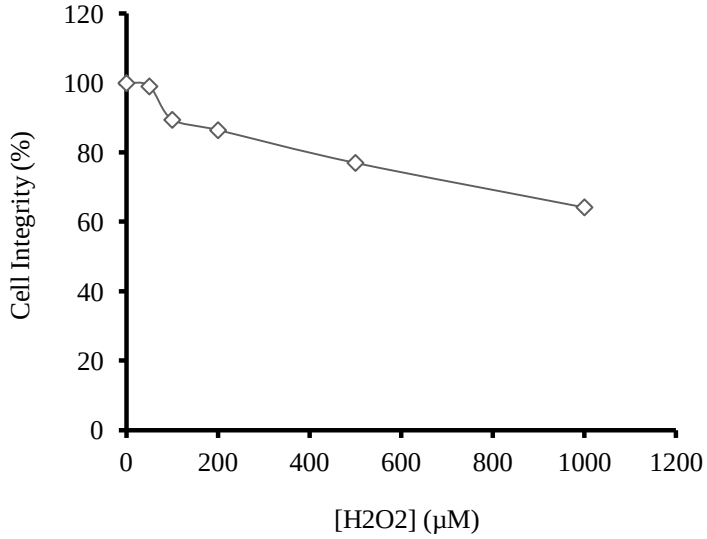


Figure 2-2. Example of MTT assay. HepG2 cells were incubated with a range of hydrogen peroxide ($H_2O_{2)}$ concentrations (200-1000 µM) for 12 hours. Subsequently cell viability was determined using the MTT assay, as described in this chapter.
Typical results are displayed.

2.2.3.2. Lactate dehydrogenase (LDH) assay

The LDH activity test is based on the measurement of the cytosolic enzyme lactate dehydrogenase. Presence of the enzyme in the extracellular space is indicative of cell

leakage due to cell damage. Thus the more enzyme is measured in the medium the more cells have been damaged (Keilhoff and Wolf, 1993).

For the quantification of the cytosolic LDH, the lactate dehydrogenase activity assay kit from Roche Applied Science was used. Catalyst lyophilized powder, provided from the manufacturer, was reconstituted in 1 mL H_2O. For the cytotoxicity test, the reconstituted catalyst and the dye solution were mixed (1:45).

For the test, cells grown to confluence in 6 well plates were incubated with a range of concentrations of the compounds for which cytotoxicity had to be assessed. The incubation conditions (temperature and time of incubation) were selected to be the same as in the final experiments. The assay was carried out in 96 well-plates as follows. 100 µL cell culture medium were collected and mixed with 100 µL catalyst + dye solution.

Incubation was carried out for 10 sec at RT under shaking condition and subsequently for 10 min at RT without shaking. 50 µL of stopping solution were added and absorbance was measured at 492 nm in a 96 well plate reader (fig. 2-3).

The following controls were used.

1. Cell-free cell culture medium. For background values.
2. Untreated cells, where no stressors had been applied. For spontaneous LDH release in the medium.
3. Lysed cells. Cells were treated with lysing buffer so that total cell lysis was induced (100 % LDH release).

Controls were prepared as shown in the table below (table 2-3).

Table 2-3. Preparation of controls used in the LDH activity assay.

	Ctr 1	Ctr 2	Ctr 3
Cell-free medium	100 µL	50 µL	50 µL
Cells	-	50 µL	50 µL
Lysis buffer	-	-	5 µL

Cell damage was measured as follows:

Cell damage (%) = (Experiment value – control 2) / (control 3 – control) x 100

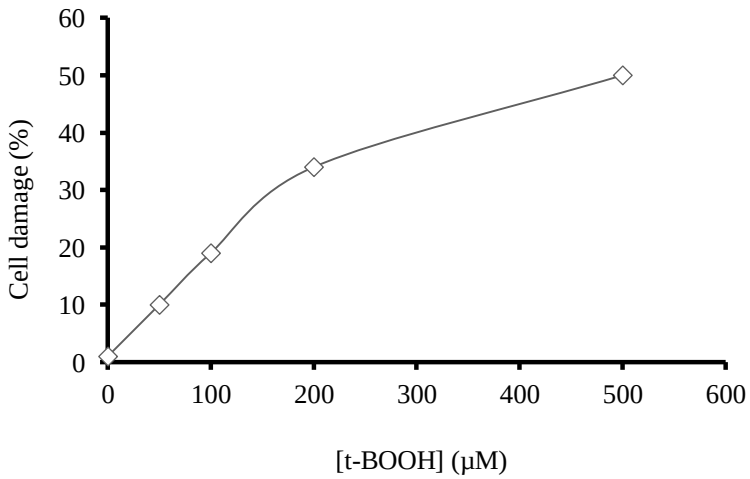


Figure 2-3. Example of LDH assay. HepG2 cells were incubated with a range of tert-*butyl* hydroperoxide (t-BOOH) concentrations (50-500 µM) for 8 hours. Subsequently cellular damage was determined using the LDH assay, as described in this chapter.
Typical results are displayed.

2.3. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Qualitative RT-PCR is a technique used to amplify specific segments of DNA.

RNA is initially extracted from the cells. Subsequently complementary DNA (cDNA) is transcribed from the RNA. This step is done through reverse transcription, using the enzyme Taq DNA polymerase. The cDNA is finally used as a template for amplification of the genes of interest. Two primers, short DNA fragments, complementary to the 3' and 5' ends of the gene of interest are used as starting points for DNA synthesis.

During synthesis of the cDNA, a reporter dye and a quencher dye are located respectively at the 5' and 3' end of a short probe. This probe anneals downstream from one of the primers. Proximity between the reporter and the quencher prevents significant emission from the reporter, due to fluorescence resonance energy transfer (FRET). When the Taq DNA polymerase reaches the probe, it acts as endonuclease on the probe. It cleaves it, causing separation of the quencher from the reporter. This causes increase in the energy emission of the reporter.

With each PCR cycle a probe is cleaved from the Taq DNA polymerase. This results in an increase of the emitted fluorescence proportional to the amount of amplified segments (Gelmini *et al.*, 2001). The RT-PCR is used to establish changes in the expression of selected genes.

2.3.1 RNA extraction

Before RNA extraction, cells were washed twice with ice-cold PBS. Total RNA was extracted using the RNAqueous® kit according to the protocol supplied from the manufacture as follows.

Cells were harvested through incubation with 250 µL/well lysis buffer for 8 min on ice and mixed with an equal volume of 70 % ethanol. Samples were shredded using a syringe in order to fragment the DNA. Samples were subsequently filtered using silica filters, allowing

the RNA to bind to the filters. A series of washes was performed to purify the RNA. RNA was finally eluted and stored at -80 °C until use.

RNA was quantified using a NanoDrop, measuring absorbance at 260 nm.

Measurements at 280 and 230 nm were also performed to assess RNA purity. 260/280 and 260/230 nm ratios were used to assess contamination from proteins, phenols and other organic components.

Values between 2.0 and 2.1 were considered to indicate acceptably pure RNA.

2.3.2. cDNA synthesis

The High Capacity RNA-to-cDNA Master Mix was used to synthesize cDNA.

Each reaction tube contained the following components:

10 μL Buffer Mix + 1 μL Enzyme Mix + 9 μL diluted RNA (= 1 μg)

The program used for cDNA synthesis was the following:

60 min at 37 °C, 5 min at 95 °C, with subsequent cooling down to 4 °C.

2.3.3. RT-PCR and gene amplification analysis

Standard curves for the genes of interest were made using a series of dilutions of the cDNA. GAPDH was selected as endogenous control (Fig. 2-4 and 2-5). The gene of interest and the endogenous control were labeled with VIC and FAM dyes respectively.

The optimal cDNA dilution was chosen using the standard curve, so that the threshold cycle (Ct) value would be between 15 and 40 cycles.

The Ct value is the number of PCR cycles necessary for the fluorescence of the report dye to exceed the background signal at a given threshold.

The Ct value inversely correlates to the amount of target gene in the sample. This value gives an overall indication of the amount of target gene in the sample, (McKenney *et al.*, 2014).

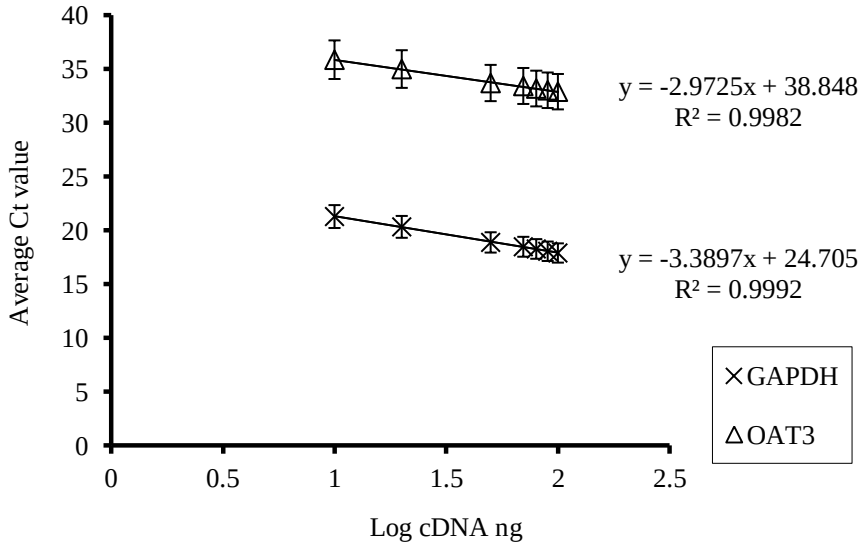


Figure 2-4. Standard curve for OAT3 gene expression. Gene expression was evaluated in HepG2 cells (see chapter 4). RNA was extracted at day 3 post-seeding, when cells reached confluence. RNA was quantified and a range of dilutions were prepared (10-100 ng). RT-PCR was carried out for each dilution. GAPDH was used as the endogenous control. The results reported represent average of triplicates ± St. Dev.

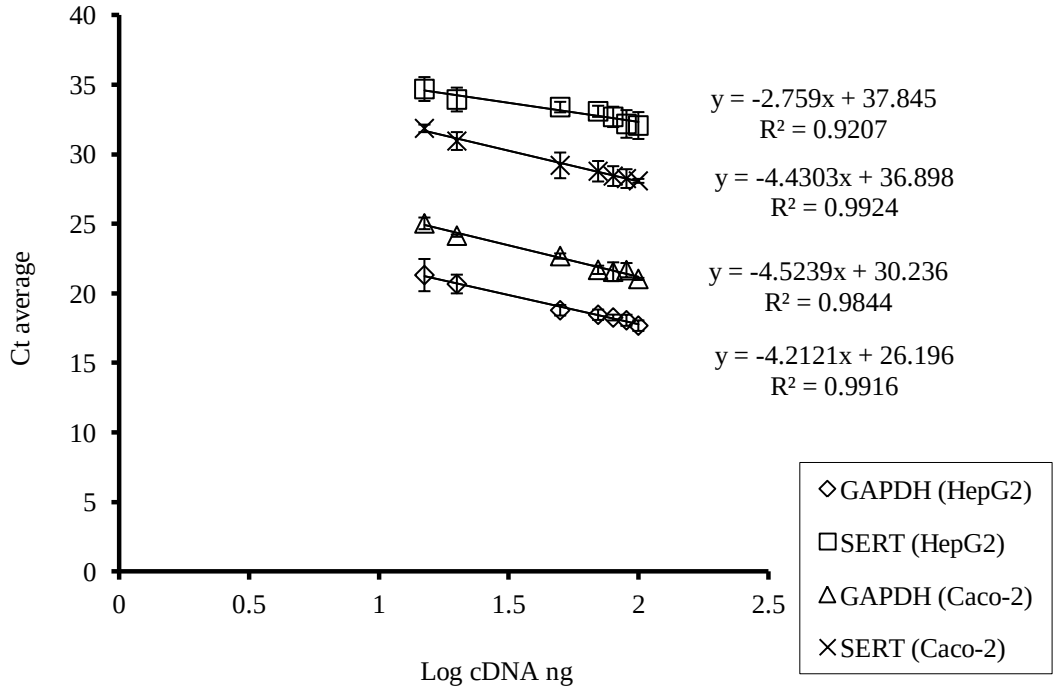


Figure 2-5. Standard curves for SERT gene expression. Gene expression was evaluated in Caco-2 and HepG2 cells, for comparative analysis (see chapter 5). RNA was extracted from Caco-2 cells at day 14 post-confluence and at day 1 post-confluence from HepG2 cells. RNA was quantified and a range of dilutions were prepared (10-100 ng).

RT-PCR was carried out for each dilution. GAPDH was used as the endogenous control.

The results reported represent average of triplicates ± St. Dev.

Each reaction tube contained the following components:

1 µL GAPDH + 1 µL OAT3 primers + 10 µL Master Mix + 8 µL diluted cDNA.

The PCR program selected was the following:

5 min at 50 °C, 10 min at 95 °C for primer annealing, 40 cycles of 15 sec at 95 °C and 1 min at 60 °C.

Results were analyzed using StepOne software 2.1.

2.4. Cellular uptake assays

2.4.1. Functional modulation of transporters

2.4.1.1. Functional modulation of OAT3 in HepG2 cells

Assessment of functional modulation of OAT3 was carried out by measuring uptake of a selected model substrate for OAT3. The selected model substrate was 5'-carboxyfluorescein (5'-CF) (Vallon et al., 2008). The aim was to establish whether pre-incubation with selected stressing agents would modulate OAT3, resulting in increase/decrease of 5'-CF uptake.

The following stressing agents were individually tested: hydrogen peroxide (H_2O_2), tert-*butyl* hydroperoxide (tBOOH) and ethanol (EtOH).

The protocol used had been optimised in a previous work (Wong *et al.*, 2011c).

Uptake was carried out in 12 well plates. Transport medium consisted of Hbss, supplemented with 1.8 mM $CaCl_2$ for reinforcement of the tight junctions. The pH was adjusted to the physiological value of 7.4.

Cells were washed once with warm transport medium and incubated at 37 °C with each stressing agent at a range of concentrations, for either 8 or 12 h.

Incubation times and concentrations were the following: 8 h with H_2O_2 (50-200 μM), 12 h with H_2O_2 (25-100 μM), 8 h with t-BOOH (50-100 μM), 12 h with t-BOOH (25 μM), 12 h with EtOH (0.5-1 mM).

The stressing agent concentrations were selected after cytotoxicity assessment of each compound, so that cell viability/cellular integrity ≥ 85%.

After incubation with the stressing agent, the transport medium containing the stressor was removed and replaced with fresh transport medium containing 100 μM 5'-CF.

5'-CF was prepared by initially dissolving the compound in DMSO to make a stock solution (100 mM). The stock solution was subsequently diluted in the transport medium (final DMSO ≤ 0.5%) to obtain the wanted concentration (100 μM).

Cells were incubated for 10 min with 5'-CF and subsequently washed with ice cold transport medium. Cell lysis was carried out using 80 % methanol for 1 h in the dark. After lysis each well content was collected. Quantification of the intracellular 5'-CF content was carried out through HPLC.

2.4.1.2. Modulation of SERT in Caco-2 cells

For uptake modulation of SERT, the transport medium was prepared in ultra-pure water as follows (in mM): 5.4 KCl, 0.44 K_2HPO_4, 4.2 $NaHCO_3$, 137 NaCl, 0.35 Na_2HPO_4, 5.5 D-glucose, 1.8 $CaCl_2$.

The substrate (serotonin, 5-HT) was present as a mixture of both 5-HT and [^{3}H]-5-HT to a final concentration of 0.2 μM.

The protocol was carried out according to previous research (Mendoza et al., 2009).

A stock solution of 5-HT (1 mM) was prepared by dissolving 5-HT in DMSO. Subsequently the stock solution was diluted in the transport medium (final DMSO % ≤ 0.1), to obtain the wanted concentrations.

Cells were pre-incubated with substrate-free transport medium for 30 min at 37°C, to reinforce the tight junctions. Cells were quickly washed twice with 1 mL warm substrate-free transport medium and incubated for 6 min at 37 °C with medium containing the substrate and 0.1 % BSA. BSA was used to minimize non-specific transport.

After 6 min transport was stopped by removing the medium and rinsing the cells twice with 1 mL ice cold medium containing 0.1 % BSA.

Finally, cells were solubilised for 30 min in 1 mL in 1 M NaOH. The whole lysates were collected and neutralised using 1 M HCl. Samples were mixed with scintillation fluid before measuring the radioactivity (β emission of tritium), using a Tri-Carb 1600TR scintillation counter.

2.4.1.2.1. Serotonin uptake optimisation

Preliminary time-linearity of serotonin uptake was determined. This was done by incubating the cells with 0.2 μM of substrate mixture for a time range (1-15 min).

Concentration-dependence of serotonin uptake was also assessed. This was done by incubating cells for 6 min with a range of substrate concentrations (0.1-5 μM).

Finally, the role of sodium in serotonin uptake was preliminary determined. This was done by measuring serotonin uptake in sodium-containing medium in sodium-depleted medium respectively. In the sodium-depleted medium, sodium was replaced with potassium.

2.4.1.2.2. Serotonin uptake modulation

It was assessed whether coffee and green tea whole extracts and individual coffee and green tea compounds modulated serotonin uptake through SERT.

Cells were incubated for 6 min at 37 °C with each compound.

Coffee and green tea whole extracts were tested at concentrations between 2 and 30 mg/mL. Coffee whole extracts were prepared by dissolving soluble coffee powder in warm HBSS. pH was adjusted to the physiological value of 7.4. Solution was vigorously vortexed for 2 min and centrifuged for 5 min at 17,000 g. The supernatant was collected and the pellet discarded. Supernatant was filtered and applied to cells.

Green tea whole extracts were prepared by dissolving green tea soluble powder in warm HBSS. Solution was vigorously vortexed for 3 min and then applied to cells.

2.4.1.2.2.1. Modulation of serotonin uptake using individual coffee compounds

The following individual coffee compounds were tested: caffeine, caffeic acid (CA), ferulic acid (FA), 3, 4-dimethoxycinamic acid (3,4-DMCIN), 5-feruoylquinic acid (5-FQA), 3-caffeoylquinic acid (3-CQA), 5-affeoylquinic acid (5-CQA), 3,4-dicaffeoylquinic acid (3,4-diCQA), 3,5-dicaffeoylquinic acid (3,5-diCQA). Initially all compounds, except for caffeine, were tested at physiological concentrations (≤ 3 mM). Subsequently the following compounds were tested at concentrations equivalent to a strong cup of coffee (15 mg/mL): caffeine: 23 mM, CA: 23 μM, FA: 6 μM, 3,4-DMCIN: 02.7 μM, 5-FQA: 400 μM, 3-CQA: 0.9 mM and 5-CQA: 3.4 mM. Imipramine was used as positive control and tested at concentrations between 5 and 15 μM.

Finally FA and 5-FQA were tested to assess whether they inhibited uptake in a competitive manner. They were tested at 1-3 mM and 0.5-2.5 mM respectively, co-incubating with a range of serotonin concentrations (between 0.05 and 1 μM).

2.4.1.2.2.2. Modulation using individual green tea compounds

The following individual green tea components were tested: epigallocatechin gallate (EGCG), epicatechin (EC), epigallocatechin (EGC) and epicatechin gallate (ECG). They were initially tested at physiological concentrations between 100 and 1000 μM. Subsequently EGCG, EC, EGC and ECG were used at supplement concentrations, equivalent to 7 cups of green tea (11.6, 2.4, 12 and 2.15 mM, respectively).

2.4.1.2.3. Radiochemistry

Intracellular tritium-labeled serotonin ([^{3}H]-5-HT) was measured to assess functional modulation of SERT, in response to coffee and green tea whole extracts and individual compounds.

The amount of radio-labeled serotonin to be used was calculated on the basis of the information provided from the manufacturer (activity amount: 250 µCi, concentration: 1µCi/µL, specific activity: 82.2 Ci/mmol).

The final serotonin concentration wanted was chosen as 0.2 µM, according to published research (Mendoza et al., 2009).

2.4.1.2.3.1. Radioactive sample preparation and radioactivity measurement

Samples were prepared for radioactivity measurement as follows.

1 mL of neutralised cell lysates was mixed with 10 mL of scintillation fluid. Beta- emissions were counted over a period of 10 min in a scintillation counter. Radioactive count was given from the counter as count of emission per minute (CPM) and then converted into degradation per minute (DPM).

In every experiment, scintillation counter efficiency was evaluated using a ^{3}H standard. Comparison between the predicted value (given by the manufacturer) and the obtained value was carried out as follows:

Detector Efficiency = CPM value obtained for the standard/CPM predicted for the standard

The CPM was converted into DPM using the following formula:

$DPM = (CPM_{sample} - CPM_{background})/\text{Detector Efficiency}$

2.4.2. Cellular uptake of kaempferol-3-*O*-glucuronide

The aim was to determine whether kaempferol-3-*O*-glucuronide (k-3-*O*-g) uptake in HepG2 cells mainly occurred in a transporter-mediated manner or it relayed on passive diffusion mechanisms.

The experiment was carried out according to previous published research (Wong *et al.*, 2012) as follows.

Uptake was evaluated using 12 well plates. Transport medium was prepared in ultra-pure water adding the following components (in mM): 4 KCl, 0.44 K_2HPO_4, 4.2 $NaHCO_3$, 137 NaCl, 0.35 Na_2HPO_4, 5.5 D-glucose and 1.8 $CaCl_2$.

Cells were washed once with warm transport buffer and incubated with k-3-*O*-g under the conditions described below.

K-3-*O*-g uptake was carried out for 10 min at 37 °C using a range of k-3-*O*-g concentrations (50-200 µM) or for 10 min under different temperatures (37°C and 4°C) using 200 µ k-3-*O*-g. The aim was to measure concentration- and temperature-dependence of k-3-*O*-g uptake, respectively.

Time-linearity of the uptake was also evaluated. Cells were incubated for a time range (10-30 min) with 200 µM k-3-*O*-g.

K-3-*O*-g had initially been dissolved in DMSO to prepare a stock solution (200 mM). The stock solution was subsequently diluted in the transport medium to obtain the wanted concentrations (final DMSO $\leq$ 0.5 %).

After incubation, cells were washed twice with ice cold transport buffer supplemented with 0.2 % bovine serum albumin (BSA). Cells were washed once with ice cold transport buffer without BSA. Cells were scraped in 0.5 mL 50 % methanol and collected for measurement of the intracellular k-3-*O*-g through HPLC.

2.4.2.1. Sample deproteination and concentration

Proteins are large molecules, whose presence in the sample could cause damage to the HPLC systems. For this reason samples were deproteinated as follows.

0.5 mL of each sample (in 50 % methanol) were sonicated for 5 min, in order to complete cellular membrane breakage. 1 mL ice cold acetone was added and samples were frozen for 1 h at -20 °C. Acetone affects the overall protein solubility in water, reducing it and causing precipitation of the proteins (Simpson and Beynon, 2010).

Lysates were thawed and centrifuged for 5 min at 17,000 g. Supernatant and pellet were separated. All the supernatant was collected and evaporated under vacuum at 30 °C. Subsequently samples were re-dissolved in 100 μL 95 % (v/v) acetonitrile for quantification of k-3-*O*-g, through HPLC analysis. Daidzin (50 μM) was added in the samples, before HPLC analysis, and used as internal standard. Optimisation was previously carried out to assess the extraction efficiency, which resulted to be ≥ 98 %.

The pellets, containing the precipitated proteins, were used for total protein quantification, using the Bradford assay.

2.4.2.1.1. Protein quantification using the Bradford assay

The Bradford assay is a colorimetric assay used to quantify the total protein amount in a sample. The assay is based on the absorbance shift of the dye Coomassie Brilliant Blue G-250, commonly known as Bradford reagent. When the dye binds to proteins, it changes from the red form to the blue form. The more proteins are present in the sample, the more dye-protein complexes will be present. The blue form of the dye maximally absorbs at 595 nm, thus proteins can be quantified measuring absorbance at 595 nm (Aminian *et al.*, 2013).

The assay was performed in 96 well plates.

For the standard curve, between 0.1 and 1.4 mg BSA were dissolved in 1 mL 0.1 M NaOH. For protein quantification in the samples, pellets were dissolved in 0.5 mL 50% NaOH and 50% HCl. Before absorbance measurement, 5 μL of BSA standard or protein sample were mixed with 0.25 mL of Bradford reagent and incubated at room temperature for 15 min. Protein quantification was performed at 595 nm, using a Multiskan Ascent 96-well plate reader.

The assay was used to assess protein content variation among wells. Either HepG2 or Caco-2 cells were seeded in 12 well plates and grown to confluence. Subsequently the cells were lysated and the Bradford assay performed for each well. The average protein amount for HepG2 cells resulted to be 1.16 mg/ mL, with a standard deviation of 0.04. The average protein content for Caco-2 cells resulted to be 0.98 mg/mL, with a standard deviation of 0.02. The assay showed that the protein content variation among wells was very small, both when working with HepG2 and Caco-2 cells.

An example of BSA standard curve is shown below in figure 2-6.

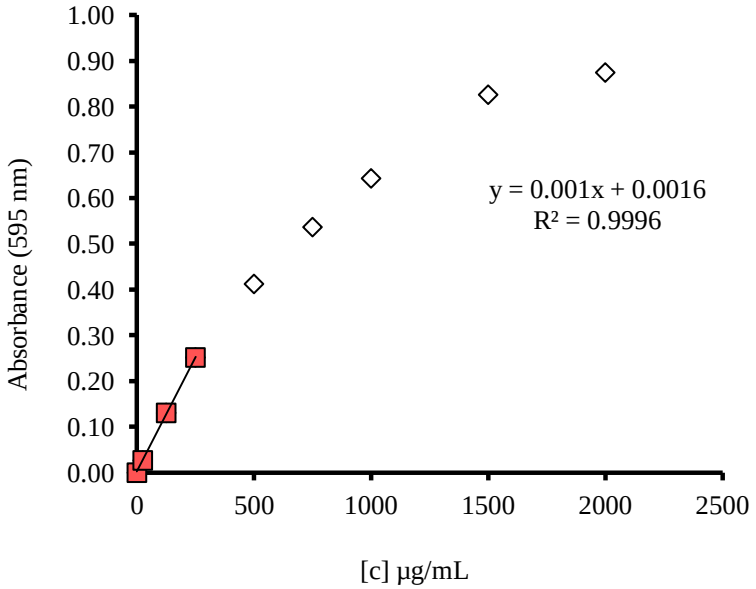


Figure 2.6. Example of Bradford assay standard curve. Between 0.1 and 1.4 mg BSA were dissolved in 1 mL 0.1 M NaOH. 5 µL of BSA sample were mixed with 0.25 mL Bradford reagent. Protein quantification was made at 595 nm, using a plate reader.

2.5. High-performance liquid chromatography (HPLC) analysis

HPLC is an analytical chemistry technique, used to quantify compounds in a mixture.

HPLC is based on a system of under pressure pumps. Selected organic solvents (mobile phase) are pumped, along with the mixture sample, through a column (stationary phase) packed with solid absorbent material.

The solvents and the parameters used to perform the elution are chosen mainly according to the type of packaging material in the column and the nature of the mixture to analyse. When the mixture to be analysed is pumped through the column, each component present in it interacts with the packaging material of the column in a different way, according to its chemical nature and structure. This results in different compounds eluting from the column at different times (retention times).

Each compound has its own retention time, strictly associated to the solvent(s), elution method and column used in the elution. As long as pure standard compounds are available, it is possible to identify the specific retention time for the compound of interest and use it to analyse and quantify the same compound in a mixture (Kormany *et al.*, 2013).
For all my analysis, the stationary phase consisted of a 4.6 x 50 mm column, C_{18}, 1.8 µM particle packed.

2.5.1. Quantification of intracellular 5'-carboxyfluorescein

Intracellular 5'-carboxfluorescein (5'-CF) was quantified using a 10 min isocratic elution run. The mobile phase consisted of 20 mM Tris-HCl (pH 8) and 20 % methanol in water. The flow rate was 0.5 mL/min. Detection of 5'-CF was carried out at 492 and at 517 nm. Quantification was performed based on standard curves, prepared using authentic standards.

2.5.2. Quantification of kaempferol-3-*O*-glucuronide

For quantification of the intracellular k-3-*O*-g a 33 min run was used. Flow rate was 0.5 mL/min. The mobile phase was made by solvent A and solvent B. A and B were respectively 5 % (v/v) acetonitrile in water and 95 % (v/v) acetonitrile in water, each containing 0.1 % formic acid. Solvent B gradient was started at 5 %, linearly increased to 10 % over 5 min. Subsequently B was increased to 40 % in the next 4 min and kept at 90 % for 2 min. Finally re-equilibration was carried out at 5 % for 4 min. The detection was carried out between 250 and 340 nm, based on standard curves prepared using authentic standards.

2.5.3. Quantification of chlorogenic acids, after coffee hydrolysis with the enzyme chlorogenate esterase

The aim was to better understand the role that chlorogenic acids present in coffee play in modulating serotonin uptake through SERT. In order to do that, coffee whole extracts (15 mg/mL) were hydrolysed using the enzyme chlorogenate esterase. After hydrolysis, the chlorogenic acid content remaining were quantified through HPLC.

Whole coffee extracts (15 mg/mL) were incubated with the enzyme chlorogenate esterase. This was done in order to hydrolyse the majority of the chlorogenic acids present in coffee. To determine what percentage of the total amount of chlorogenic acids had been hydrolysed, HPLC analysis was performed, quantifying the chlorogenic acids in hydrolysed and un-hydrolysed coffee extracts.

In a subsequent experiment Caco-2 cells were treated with hydrolysed and un-hydrolysed coffee respectively and serotonin modulation was assessed. Comparison between the effect of coffee containing chlorogenic acids and coffee were the majority of the chlorogenic acids had been hydrolysed was carried out.

Analysis of coffee chlorogenic acids was carried out using a 68 min run with 0.26 mL/min flow rate. The solvents were 5 % (v/v) acetonitrile in water (A) and 95 % (v/v) acetonitrile in water (B), each containing 0.1 % formic acid. Elution was started with 0 % solvent B, maintained at 0 % for 17 min. Percentage of B was subsequently increased to 25 over the next 34 min. Percentage of B was immediately increased to 100 and kept at 100 for 5 min. Finally column re-equilibration was carried out for 12 min using 0 % solvent B. The detection was carried out between 250 and 340 nm, using standard curves prepared with authentic standards.

2.5.3.1 Coffee hydrolysis with chlorogenate esterase

2.5.3.1.1. Assessment of Chlorogenate esterase efficacy

Optimisation was carried out to determine the amount of chlorogenate esterase to be used for coffee hydrolysis.

The aim was to use an amount of chlorogenate esterase, so that the majority of the chlorogenic acid content would be hydrolysed.

The enzyme activity for a selected enzyme represents a measure of the hydrolyzing potency of the enzyme. It represents the quantity of active enzyme present. The enzyme activity depends on the conditions used to perform the assay (Frei, 1970).

When an enzyme is purchased, the manufacturer specifies the minimum enzyme activity for the product and the conditions under which this measurement was initially made. The manufacturer does not provide the exact enzyme activity. This must be calculated from the user and re-calculated whenever new experimental conditions are selected.

Enzyme activity tends to decrease over time. For this reason, it is also important to assess it at regular time intervals.

Assessment of the enzyme activity was carried out based on the guidelines specified from the manufacturer. The main steps of the assay are reported below:

5-*O*-caffeoylquinic acid + H_2O + chlorogenate esterase $\longrightarrow$ caffeic acid + quinic acid

- A phosphate buffer (here called reagent A) was prepared mixing 50 mM sodium phosphate dibasic (Na_2HPO_4) and 50 mM potassium phosphate monobasic (KH_2PO_4). The buffer pH was adjusted at pH 6.5.

- 26 mg of the substrate 5-*O*-caffeoylquinic acid were dissolved in 0.5 mL methanol. The 5-*O*-coffeoylquinic acid solution was diluted using 45.5 mL of reagent A to obtain a 1.5 mM solution (here called reagent B).
- Reagent C (80 % methanol solution) was prepared mixing 20 mL of water and 80 mL methanol 100 %.
- The enzyme solution was prepared immediately before use as follows and kept on ice until use. The enzyme was dissolved in ice-cold reagent A, to a final volume activity of 0.3 U, equivalent to 20 mg powder (calculated on the base of the information provided by the manufacturer, that enzyme activity was ≥ 15 U/g).

One unit (U) of activity is defined as the enzyme amount that performs hydrolysis of 1 µmol 5-*O*-caffeoyl quinic acid per minute at pH 6.5 and temperature of 30 °C.

The enzyme assay was performed in triplicate as described below:

a. 0.5 mL of enzyme solution was put in a 15 mL centrifuge tube,
b. solution was equilibrated at 30 °C in water bath,
c. 0.03 mL of the solution B was added to the tube. The mixture was briefly vortexed and incubated at 30 °C for further 30 min in water bath,
d. reaction was stopped by adding 10 mL of solution C to a final volume of 10.53 mL,
e. measurement of the absorbance was performed using a Cecil CE 201 spectrophotometer set at 350 nm, using a pair of 1 cm^2 light path disposable cuvettes.

The blank was prepared using the above protocol but inverting the order of point c. and d.

The measured absorbance was used to calculate weight activity using the following equation:

$$\text{Weight activity (U/g)} = \text{Volume activity} \times \frac{1}{C}$$

Where:

$$\text{Volume activity} = \frac{[A_0 - A_s \times 0.75\ (\mu\text{mol}) \times 1\ (\text{mL}) \times \text{df}]}{[0.62 \times 0.03\ (\text{mL}) \times 30\ (\text{min})]} = A_0 - A_s \times 1.34 \times \text{df}$$

$$\text{with df} = \frac{V_f}{V_i}$$

Equation 2-1. Calculation of enzyme activity based on enzyme powder (grams).

In the equation 0.75 is the amount of 5-*O*-caffeoyl quinic acid in 0.5 mL. 0.62 represents the change of absorbance after complete hydrolysis of 0.75 µmol of substrate under experimental conditions. V_f and V_i are respectively the final (10.53 mL) and initial (0.5 mL) volume of the assay. C is the enzyme concentration (0.02 g/mL).

Calculation resulted in a weight activity of 15.5 U/g. This value was used to calculate the amount of enzyme powder required in order to have the wanted enzyme activity of 0.2 U:

$$\text{Amount of enzyme to be used (mg)} = [0.2\ (\text{U})] / [15.5\ (\text{U/g}) / 1000]$$

Equation 2-2. Calculation of enzyme powder to be used.

The wanted enzyme activity wanted was selected as 0.2 . In order to obtain this activity, 13 mg chlorogenate esterase powder were used.

2.6. Statistical analysis

Unless otherwise stated, all results are expressed as average of triplicates, resulting from performing each experiment three times. Dispersion from average is expressed using the Standard deviation (St. Dev.).

Statistical comparisons were performed by paired t-test, with a significance interval of 95 % ($P < 0.05$). Kinetic analysis of the 5-HT transport values was performed by non-linear regression, fitting the results to an equation according to the Michaelis–Menten model.

Chapter 3

A review of transporter distribution and modulation

3.1. Abstract

Transcription of both uptake and efflux transporters can be modulated from a range of conditions. Several publications describe the modulation of individual transporter genes or groups of genes. However very few papers are available that summarise the current state of knowledge available.

A comprehensive database for selected genes encoding for human transporters was created for the first time. The database was created using the high performance search engine for gene expression, Genevestigator. The database summarizes the up to date available information on selected human transporters under different conditions: biological mediators, disease, chemicals and drugs.

The following genes were investigated: *ABCC2* (MRP2), *ABCC3* (MRP3), *ABCG2* (BCRP), *SLOC1A2* (OATP1A2), *SLCO1B1* (OATP1B1), *SLCO1B3* (OATP1B3), *SLCO4C1* (OATP4C1), *SLC22A6* (OAT1), *SLC22A8* (OAT3), *SLC16A1* (MCT1), *SLC16A2* (MCT7), *SLC5A8* (SMCT1).

A database containing the available information about anatomical distribution of the selected transporters in four human organs (heart, liver, kidney and intestine) was also created.

The present review is based on the comprehensive database created and provides a vital starting point for further investigations of selected human transporters. The comprehensive database can be found in Appendix I and II.

3.2. Introduction

Transporters can be divided into uptake and efflux, according to the direction of the transport they mediate. Uptake occurs when compounds are moved towards the cell cytoplasm, while during the efflux compounds are transported out of the cell (Keppler, 2005). The activity of transporters can be modulated in two ways. In the functional modulation, a conformational change of the transporter occurs following interaction with a modulator. The conformational change results in either an increase or a decrease in the amount of substrate transported (Nunez *et al.*, 2008). In the expressional modulation, a modulator (or compounds produced in response to it into the cell) interacts with the gene promoter of the transporter, increasing or decreasing the amount of mRNA transcribed for the gene (Zembruski *et al*., 2011). In the expressional modulation, the modulator acts as a transcriptional factor (Ebert *et al.*, 2005). More details about transporter modulation are given in Chapter 1.

The possibility to control the transcription of transporters is the current focus of many studies, because it represents a tool to control and modify the cell physiology. The final aim of these studies is to optimize the biological processes, maintaining the cell into a healthy state (Wood *et al.*, 2000, Pochini *et al.*, 2013).

There are several methods to assess changes in gene transcription, ranging from techniques enabling the analysis of one gene per time to assays allowing the analysis of the whole genome in a single experiment (Wasik *et al.*, 2000, Lohr *et al.*, 2012). One method used to assess transcriptional changes is microarray analysis. DNA microarrays consist in picomole-sized DNA spots, called probes, attached to a solid surface. The probes are used to hybridize a target cDNA. Subsequently the DNA hybrids are separated and the relative abundance of the genes of interest is measured in the DNA sample. Microarray analysis is used when the

number of genes to analyse is high, because it allows analysis of a large number of genes at once, using customised arrays (Allanach *et al.*, 2008, Esparcia *et al.*, 2011).

Genevestigator is an online application, developed to provide information about gene expression coming from microarray and RNAseq experiments. Data mining systems are becoming an increasingly vital tool to collect information for research (Zimmermann *et al.*, 2005, Zimmermann *et al.*, 2008). As such, Genevestigator has been widely used for researchers to collect information on gene expression (Freitas *et al.*, 2011, Mirza *et al.*, 2012, Han *et al.*, 2012).

The information provided in Genevestigator is given for a wide range of organisms separately, so that gene expression can be investigated in a selected organism individually. A range of parameters can be selected to filter the data extraction. For example, by selecting the Anatomy tool or the Perturbation tool, the user can look at gene expression in a range of tissues or at the expression levels of the gene under a wide range of conditions, respectively.

A comprehensive database was created extracting data for six uptake and six efflux transporters. These transporters were selected as published literature exists, showing that they act as active carriers of polyphenols (Table 3-1). The information collected was used to design subsequent experiments.

Table 3-1. Examples of polyphenols acting as substrates for selected human uptake and efflux transporters.

Transporter	Example of phenolic substrate	Reference
OAT1	Ellagic acid	(Whitley *et al.*, 2005)
OAT3	Naringenin	(Hong *et al.*, 2007)
OAT1A2	Naringin	(Bailey *et al.*, 2007)
OATP1B1	Flavonoid aglycones and glycosides	(Wang *et al.*, 2005)
OATP1B3	Epigallocatechin gallate	(Roth *et al.*, 2011)
OATP4C1	Quercetin-3'-O-sulfate	(Wong *et al.*, 2012)
MRP2	Sulfate conjugates	(Brand *et al.*, 2006)
MRP3	Hesperetin glucuronide	(Brand *et al.*, 2008)
BCRP	Hesperetin	(Brand *et al.*, 2008)
MCT1	Ferulic acid	(Konishi *et al.*, 2002)
MCT7	Ferulic acid	(Konishi *et al.*, 2002)
SMCT1	Luteolin	(Passamonti *et al.*, 2009)

In the first section of this review, the anatomical distribution of each transporter of interest was assessed. In the second part the transporters of interest were investigated for their response to different conditions.

The comprehensive database, can be found in Appendix I and II. All the relevant information about the data discussed in this review (e.g. sample type, statistical relevance and material used) was extracted from Genevestigator and is provided in the databases.

3.3. Methodology

3.3.1. Tool definition

The bioinformatic tool Genevestigator (https://www.genevestigator.com/gv/) (Zimmermann *et al.*, 2008) was used to extract all the data. Each gene of interest was investigated for anatomical distribution and expressional modulation, individually.

Genevestigator provides information about gene expression in a wide range of organisms. For the creation of the present database data mining was performed for *Homo sapiens*, thus all the data discussed in the present review come from experiments carried out using human samples or human cell lines.

3.3.1.1. Data normalisation in Genevestigator

Genevestigator hosts data from multiple platforms. Data come from a variety of different experiments which use multiple types of tissues, experimental designs and data integrations. In order for all the data provided to be comparable with each other normalisation is essential. Genevestigator uses Robust Multi-array Average (RMA) normalisation, which is based on the measurement of the probe-level expression of all the arrays in a study to estimate expression values (Gentleman *et al.*, 2004). RMA includes also background correction. After normalisation, it is possible to perform meta-analysis across thousands of experiments and to integrate data from a variety of different experimental sources.

For the signal intensity values arbitrary units are used, these are scaled to the total abundance of transcripts in a sample. The background range is between 0 and 100, depending on the probe set, organism used and type of array. Signal intensity above 100 means significant presence of a certain transcript, while if values are below 100 transcripts

are considered absent, because of the impossibility to clearly discriminate from background noise.

3.3.1.2. Evaluation of the anatomical distribution

The Anatomy tool allows to look at the results of experiments obtained using healthy cultured cells and healthy human tissue samples. Cancerous tissues are excluded.
Each value displayed in Genevestigator represents the mean of all the results obtained using a certain tissue/cell type. Details on the number and type of samples used and of the standard error values are given in Appendix I.

3.3.1.3. Evaluation of the expressional modulation - choice of modulatory conditions

For the analysis of transporter modulation, results are reported in Genevestigator as the ratio between the mRNA values from the treated sample and the mRNA from the control sample, untreated.
For the comprehensive database and for the present review chapter the following modulatory conditions were selected: chemicals and drugs, disease (cardiovascular and metabolic diseases and afflictions of the gastrointestinal system) and chemical mediators (cytokines/growth factors and hormones). These conditions were chosen as they all relate to the research interests of the biochemistry group where my PhD project was carried out. The main focus of interest of the group was to better understand and characterise the link existing between the modulation of human transporters and alterations of the cell physiology, resulting from degenerative pathologies. It was also of interest to understand the

effect of human dietary compounds on human transporters, with specific focus on polyphenols, because of their proved role in protecting against degenerative disease (Botden *et al.*, 2011, Mukunda *et al.*, 2012). Chemicals and drugs have high importance in terms of cell physiology, members of these two classes strongly impact on the expression of all transporters (Rausch-Derra *et al.*, 2001). Cardiovascular and metabolic disease is strictly connected to oxidative stress, since it has been shown that oxidative stress is a major cause of degenerative disease (Usberti *et al.*, 2002, Khan, 2012). The gastrointestinal system plays a crucial role in the metabolism of all the ingested compounds (including polyphenols) and is an incredibly rich site of transporters (Chiou *et al.*, 2001). It has been shown that gastrointestinal tract disease impact on the distribution of nutrients across all the biological organs, due to transporter expression and function alteration (Kovacs et al., 2007). Finally, it has been shown that biological mediators greatly impacts on the expression of transporters across membranes and on the overall cell physiology (Attali *et al.*, 1997).

3.3.1.4. Choice of probes

When measuring gene expression using microarrays, different sets of probes are used, randomly located on the array and more or less specific for the gene of interest. In general a gene is represented by 11-16 probes of 25 nucleotides. Genevestigator presents all the available probes for the user to choose. Probes have different specificity, so that the user can choose to look at more or less specific results. When investigating gene modulation for this review, results were filtered so that the most specific available probes were selected for each gene of interest. In a very few cases, probes that were highly specific for a certain gene could not be found. In such cases, less specific probes were selected (e.g. probes specific for a motif recurrent in multiple genes within the same family).

3.3.1.4.1. Types of probes available in Genevestigator

In Genevestigator all probes carry an extension. The most specific probes, targeting nucleotidic sequences unique for a gene of interest, have either _at (anti-sense target) or _st (sense target). The _at extension is the most recurrent, while _st is found more rarely. When probes carrying _at or _st extensions existed for the genes of interest, they were the ones selected for the comprehensive database.

Below is a list of extensions, representing less specific probes compared to _at and _st (specificity decreases from top to bottom):

_s_at (or_s_st): probe sets where transcripts from different genes share common probes,

_a_at (or _a_st): probe sets recognising more than one transcript for a gene,

_x_at (or _x_st): rules of cross-hybridisation dropped, high risk of cross-hybridisation,

_g_at (or _g_st): similar genes,

_f_at (or _f_st): rules of similarity dropped, more than one gene will be recognised,

_i_at (or _i_st): sequences with less than 11 or 16 unique probes.

3.3.2. Statistical analysis

Results were considered statistically significant when the p value was < 0.05.

3.4. Review

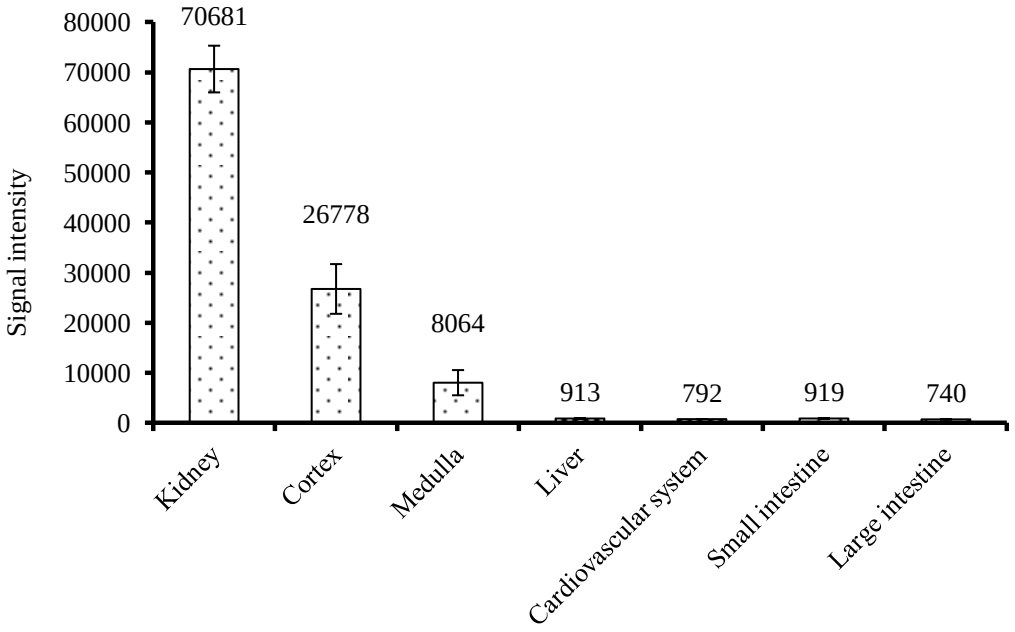
3.4.1. Tissue distribution

The tissue distribution of human transporters varies, not only from one family to another, but also within families. According to their specific function, transporters are localised on the apical or on the basolateral side of the biological membranes. The same transporter can have a different localisation depending on the tissue where it is expressed. Variation in the distribution of transporters mainly depends on substrate specificity and on the tissues where the substrates are metabolised and transported. Having information on the relative abundance and distribution of transporters is very useful to predict the function(s) of the transported substrates and the biological processes they are involved in.

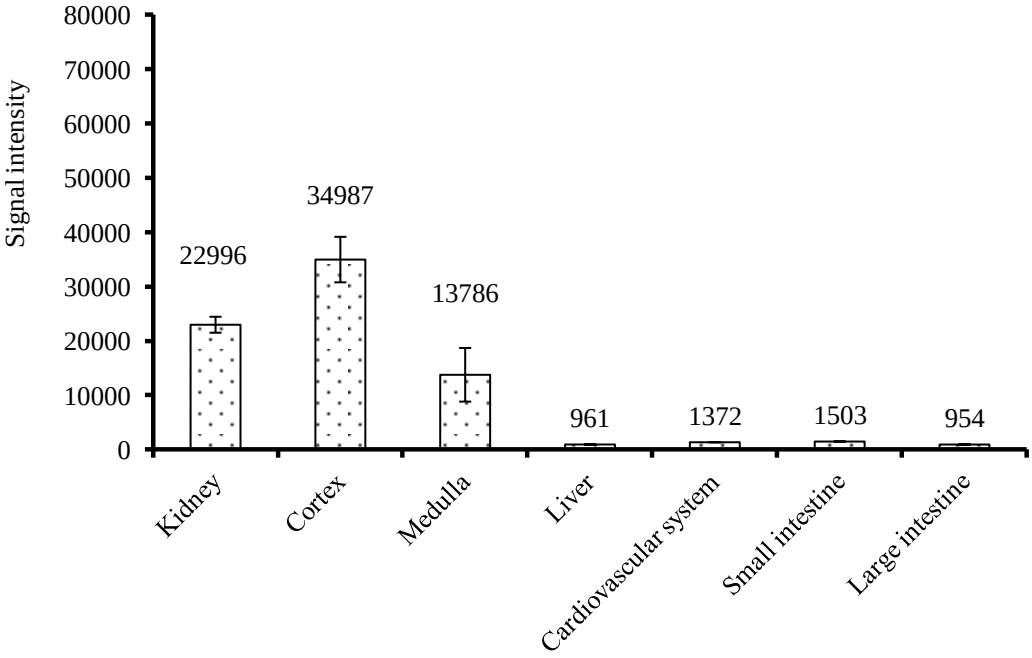
3.4.1.1. Organic anion transporters - OAT1 and OAT3

OAT1 and OAT3 showed to be abundantly expressed only in the basolateral membrane of the kidney proximal tubules. In this organ, they transport compounds from the blood into the proximal tubule cells. In kidney cells, OAT1 was expressed at higher levels compared to OAT3 (Fig. 3-1).

Figure 3-1. Anatomical distribution of the OAT transporters. Expression is shown for OAT1 (a) and OAT3 (b), using the following filling patterns: dots for the renal/urinary system, dark vertical for the liver/biliary system, small checker board for the cardiovascular system and large grid for the intestine.


Signal intensity
80000
70000
60000
50000
40000
30000
20000
10000
0
70681
26778
8064
913
792
919
740
Kidney
Cortex
Medulla
Liver
Cardiovascular system
Small intestine
Large intestine

a.

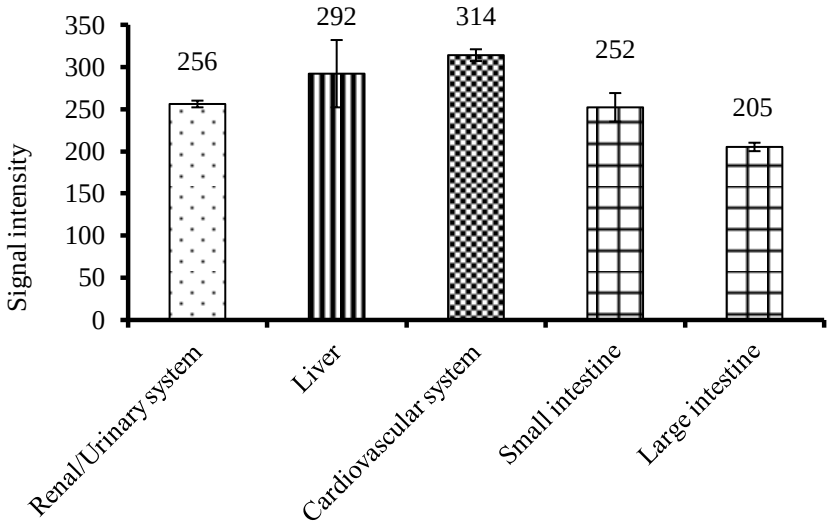

Signal intensity
80000
70000
60000
50000
40000
30000
20000
10000
0
22996
34987
13786
961
1372
1503
954
Kidney
Cortex
Medulla
Liver
Cardiovascular system
Small intestine
Large intestine

b.

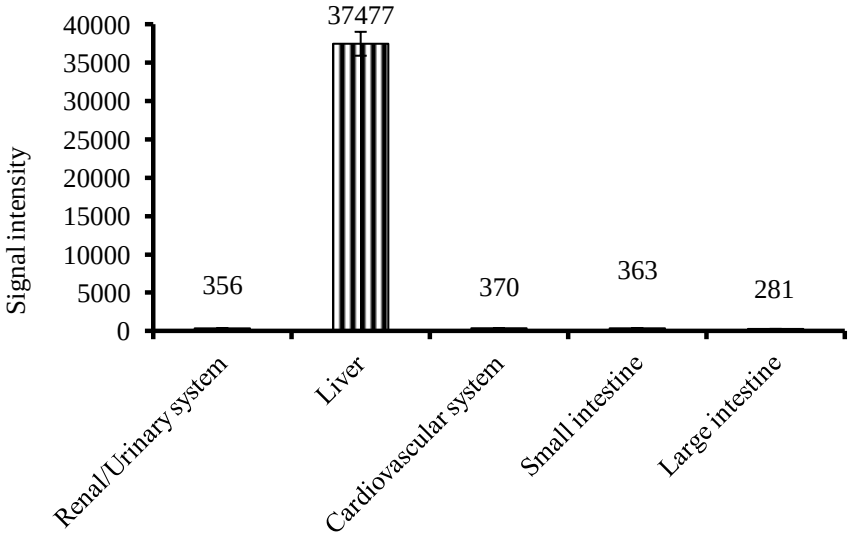
3.4.1.2. Organic anion transporting polypeptides - OATP1A2, OATP1B1, OATP1B3 and OATP4C1

Among the OATPs, OATP1A2 was the transporter with the lowest levels of expression (signal intensity was below 300 in all the investigated tissues, apart from the cardiovascular system where it was slightly above 300). OATP1A2 was the only OATP expressed in the cardiovascular system. OATP4C1 was the only OATP expressed in the kidney. Finally, OATP1B1 and OATP1B3 were only expressed in the liver, with OATP1B3 being the most abundant (Fig. 3-2).

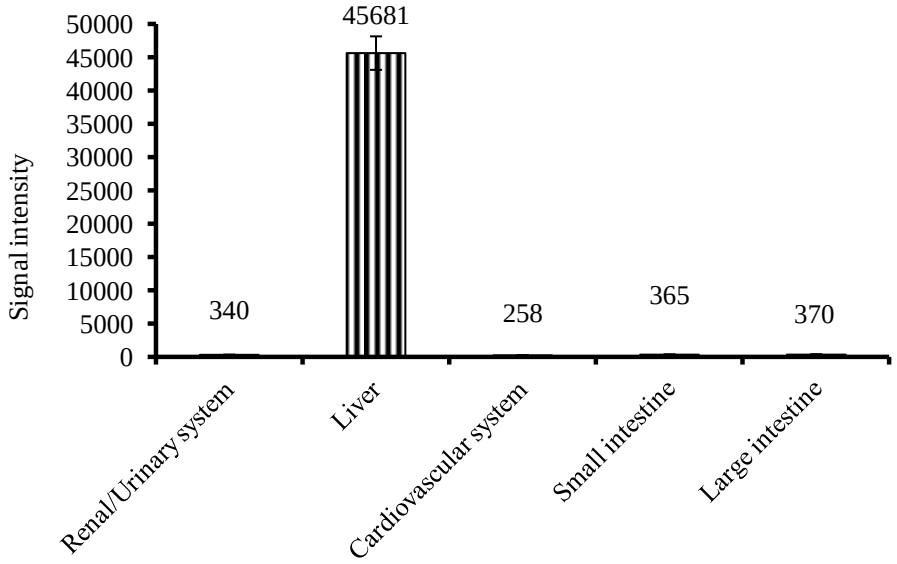
Figure 3-2. Anatomical distribution of the OATP transporters. Expression is shown for OATP1A2 (a), OATP1B1 (b), OATP1B3 (c) and OATP4C1 (d), using the following filling patterns: dots for the renal/urinary system, dark vertical for the liver/biliary system, small checker board for the cardiovascular system and large grid for the intestine.


Signal intensity
350
300
250
200
150
100
50
0
256
292
314
252
205
Renal/Urinary system
Liver
Cardiovascular system
Small intestine
Large intestine

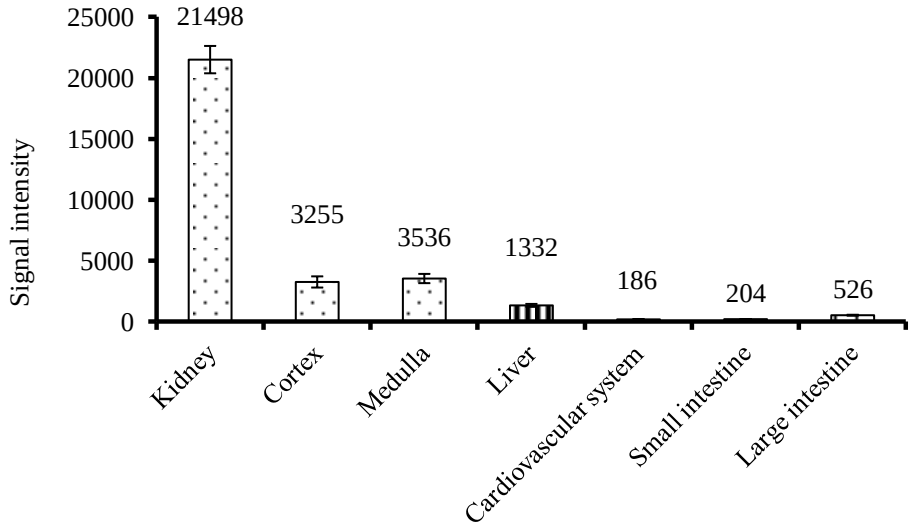
a.


Signal intensity
40000
35000
30000
25000
20000
15000
10000
5000
0
356
37477
370
363
281
Renal/Urinary system
Liver
Cardiovascular system
Small intestine
Large intestine

b.


Signal intensity
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
45681
340
258
365
370
Renal/Urinary system
Liver
Cardiovascular system
Small intestine
Large intestine

c.

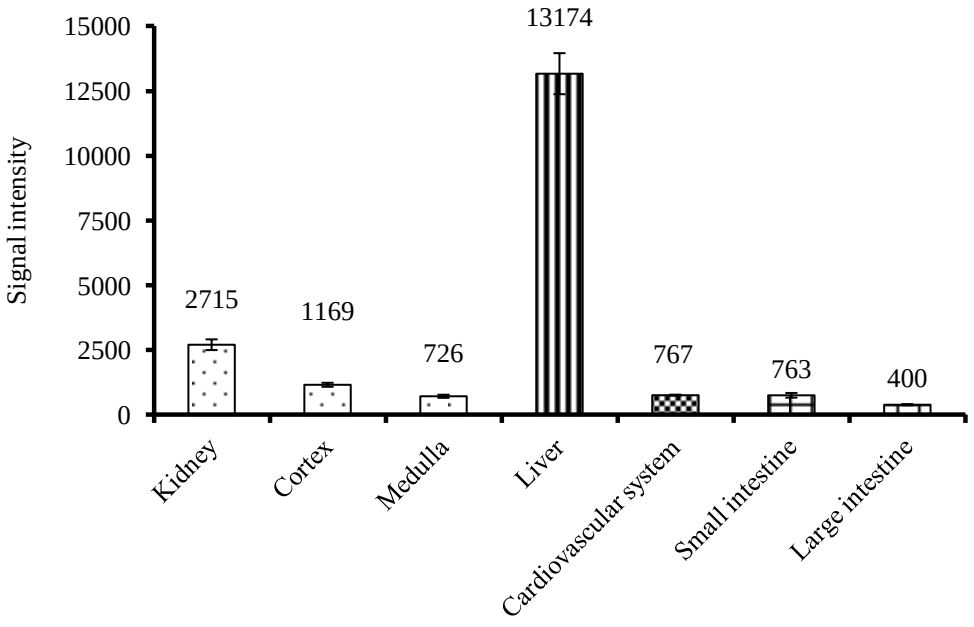

Signal intensity
25000
20000
15000
10000
5000
0
21498
3255
3536
1332
186
204
526
Kidney
Cortex
Medulla
Liver
Cardiovascular system
Small intestine
Large intestine

d.

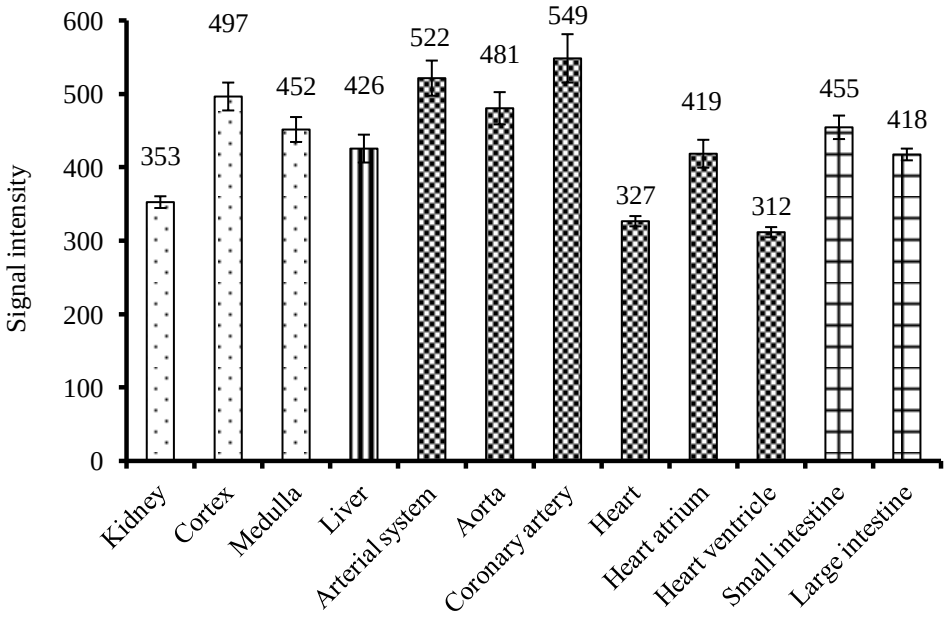
3.4.1.3. ATP-binding cassette transporters - MRP2, MRP3 and BCRP

Among the ABCs, MRP3 displayed to be ubiquitously expressed in the tissues of interest. MRP3 was the ABC expressed at the lowest levels: signal intensity was below 500 in the kidney, liver and intestine and slightly above 500 in the cardiovascular system. Among the ABCs, MRP2 was the transporter expressed in the smallest number of tissues, mainly found in liver and kidney. The main site of expression was the liver, followed by the kidney. Finally BCRP showed the highest levels of expression in the intestine, followed by the liver, which displayed slightly lower abundance compare to the intestine (Fig. 3-3).

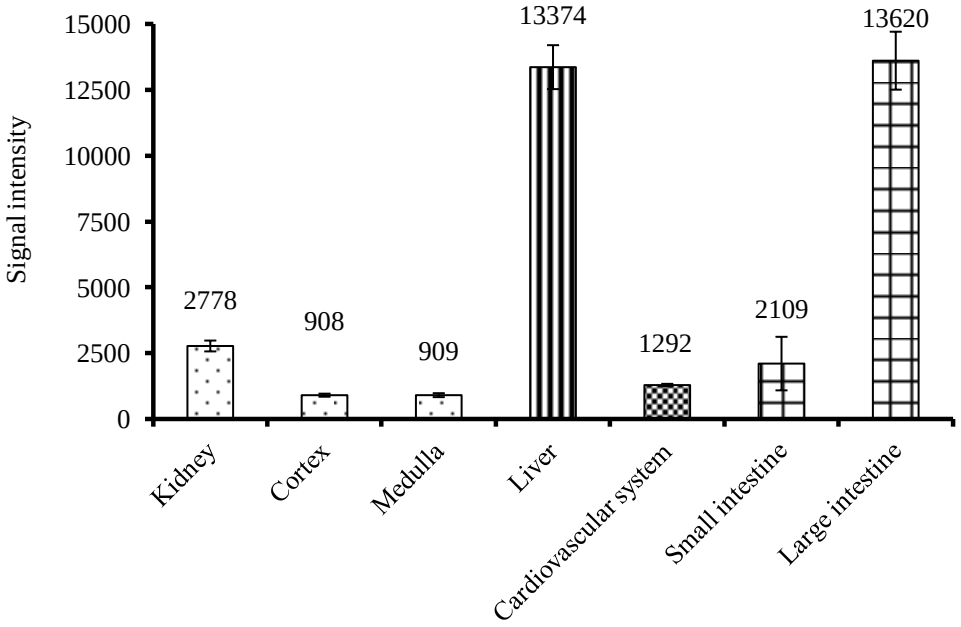
Figure 3-3. Anatomical distribution of the ABC transporters. Expression is shown for MRP2 (a), MRP3 (b) and BCRP (c), using the following filling patterns: dots for the renal/urinary system, dark vertical for the liver/biliary system, small checker board for the cardiovascular system and large grid for the intestine.


Signal intensity
15000
12500
10000
7500
5000
2500
0
2715
1169
726
13174
767
763
400
Kidney
Cortex
Medulla
Liver
Cardiovascular system
Small intestine
Large intestine

a.


Signal intensity
600
500
400
300
200
100
0
353
497
452
426
522
481
549
327
419
312
455
418
Kidney
Cortex
Medulla
Liver
Arterial system
Aorta
Coronary artery
Heart
Heart atrium
Heart ventricle
Small intestine
Large intestine

b.


Signal intensity
15000
12500
10000
7500
5000
2500
0
2778
908
909
13374
1292
2109
13620
Kidney
Cortex
Medulla
Liver
Cardiovascular system
Small intestine
Large intestine

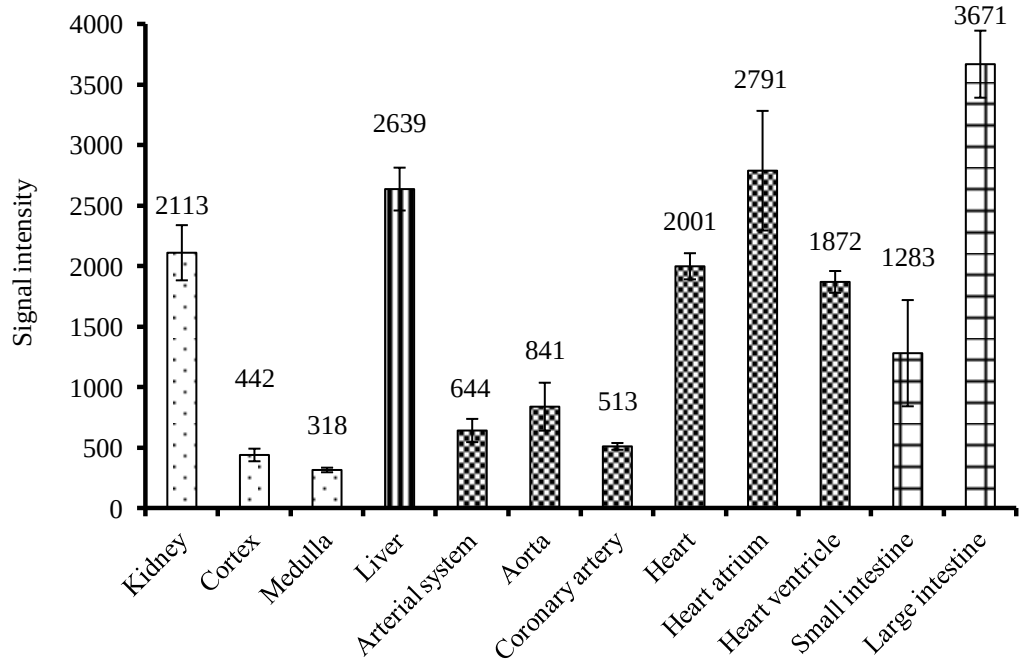
c.

3.4.1.4. Monocarboxylate transporters – MCT1, MCT7 and SMCT1 (sodium-coupled monocarboxylate transporter).

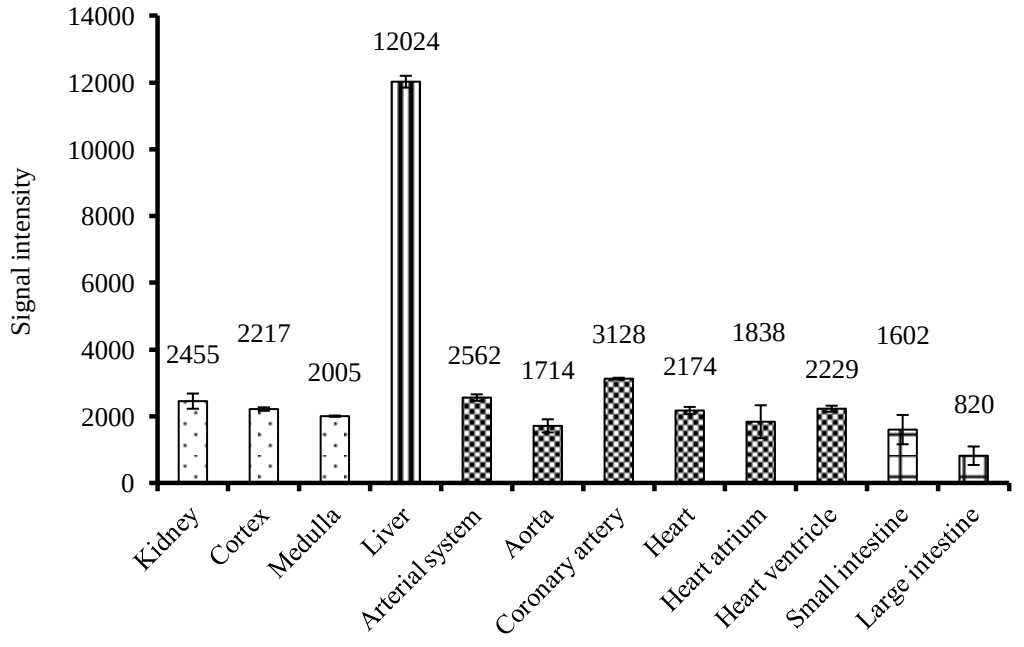
MCT1 and MCT7 were both ubiquitously expressed in the tissues of interest. The expression levels of MCT7 were higher than those of MCT1 in all the tissues investigated, except for the heart atrium, where MCT1 was more abundant than MCT7.

MCT1 displayed the highest levels of expression in the intestine followed by the heart , while the richest site of expression for MCT7 was the liver. SMCT1 also was found ubiquitously expressed, though expression was lower compared to MCT1 and MCT7. SMCT7 was most abundant in the small intestine and in the vascular components of the cardiovascular system (Fig. 3-4).

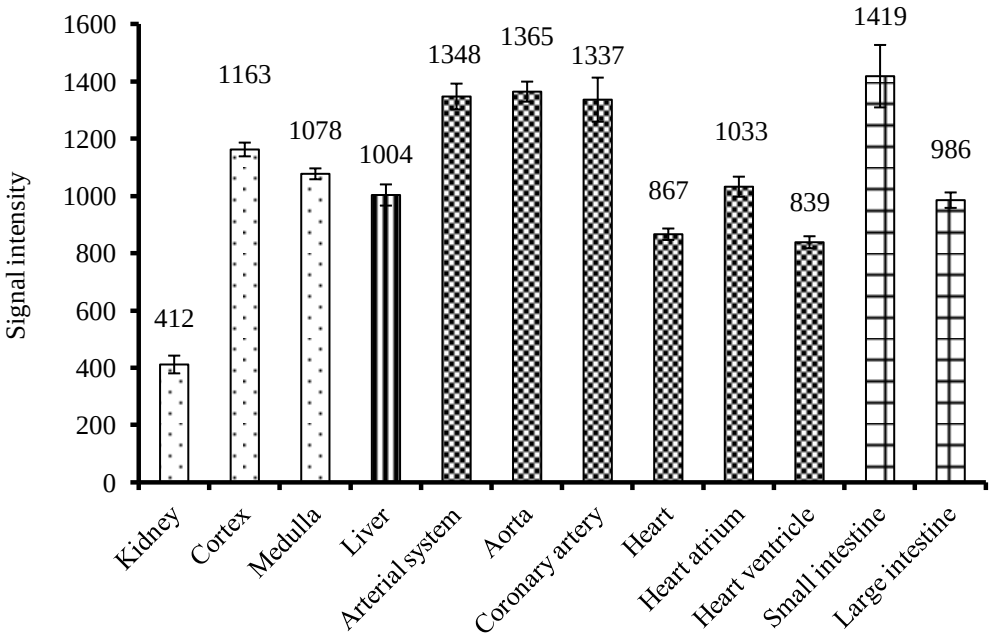
Figure 3-4. Anatomical distribution of the MCT transporters. Expression is shown for MCT1 (a), MCT7 (b) and SMCT1 (c), using the following filling patterns: dots for the renal/urinary system, dark vertical for the liver/biliary system, small checker board for the cardiovascular system and large grid for the intestine.


Signal intensity
4000
3500
3000
2500
2000
1500
1000
500
0
2113
442
318
2639
644
841
513
2001
2791
1872
1283
3671
Kidney
Cortex
Medulla
Liver
Arterial system
Aorta
Coronary artery
Heart
Heart atrium
Heart ventricle
Small intestine
Large intestine

a.


Signal intensity
14000
12000
10000
8000
6000
4000
2000
0
2455
2217
2005
12024
2562
1714
3128
2174
1838
2229
1602
820
Kidney
Cortex
Medulla
Liver
Arterial system
Aorta
Coronary artery
Heart
Heart atrium
Heart ventricle
Small intestine
Large intestine

b.


Signal intensity
1600
1400
1200
1000
800
600
400
200
0
412
1163
1078
1004
1348
1365
1337
867
1033
839
1419
986
Kidney
Cortex
Medulla
Liver
Arterial system
Aorta
Coronary artery
Heart
Heart atrium
Heart ventricle
Small intestine
Large intestine

c.

3.4.2. Transporter modulation

3.4.2.1. Modulation of transporters in response to disease, biological mediators, chemicals and drugs

Overall a variety of conditions displayed to modulate the transporters of interest. OAPT1B3 and BCRP were modulated from the highest number of conditions, among the uptake and efflux transporters, respectively. Change in their expression was triggered by 38 and 54 conditions, respectively. OATP1A2 and SMCT1 were the least affected transporters, among the uptake and efflux, respectively. A total of 15 and 25 conditions triggered modulation of the expression of these two transporters, respectively.

Among the transporters of interest, the efflux transporters resulted to be generally more responsive to modulation than the uptake transporters.

All the selected genes of interest belong to the transporter gene clusters. For this reason, generally data came from experiments were they were all investigated at once under the same modulating conditions. Since the total number of conditions used as modulators were the same for each of the transporters included in the database, the overall responsiveness of transporters is comparable.

Table 3-2 reports the number of disease, biological mediators, chemical and drugs modulating each transporter.

In the sessions below, the modulation of each class of transporters is discussed individually.

The details on the data source and samples used for transporter modulation are given in Appendix II.

Table 3-2. Number of conditions triggering transcriptional change in the genes of interest.

	Number of conditions affecting transcription				
Transporter	Chemicals and Drugs	Diseases	Growth factors and cytokines	Hormones	**Total**
OAT1	13	1	3	2	**19**
OAT3	14	4	1	1	**20**
OATP1A2	9	2	4	0	**15**
OATP1B1	15	2	3	0	**20**
OATP1B3	26	4	5	3	**38**
OATP4C1	15	6	4	5	**30**
MRP2	25	3	9	0	**37**
MRP3	23	2	7	3	**35**
BCRP	31	5	7	11	**54**
MCT1	39	3	10	9	**61**
MCT7	20	5	14	10	**49**
SMCT1	19	2	2	2	**25**

3.4.2.1.1. Modulation of the OATs (OAT1 and OAT3)

OAT1 and OAT3 showed to be modulated from a small range of conditions: a total of 19 and 20 for OAT1 and OAT3, respectively. Both responded mostly to chemicals and drugs. Not many food components were found to be used as modulators, highlighting the limited knowledge existing to date on the effect of dietary components on transporters. The only food compounds tested on OAT1 and OAT3 were apple procyanidin and curcumin. Apple procyanidin is a natural phenol, known from previous studies for its anti-inflammatory and anti-neoplastic properties (Alaikov *et al*., 2007, Malik *et al.*, 2009). Both compounds were individually used to treat human proximal tubule cells and showed no effect on the expression of OAT1 and OAT3 (data not shown).

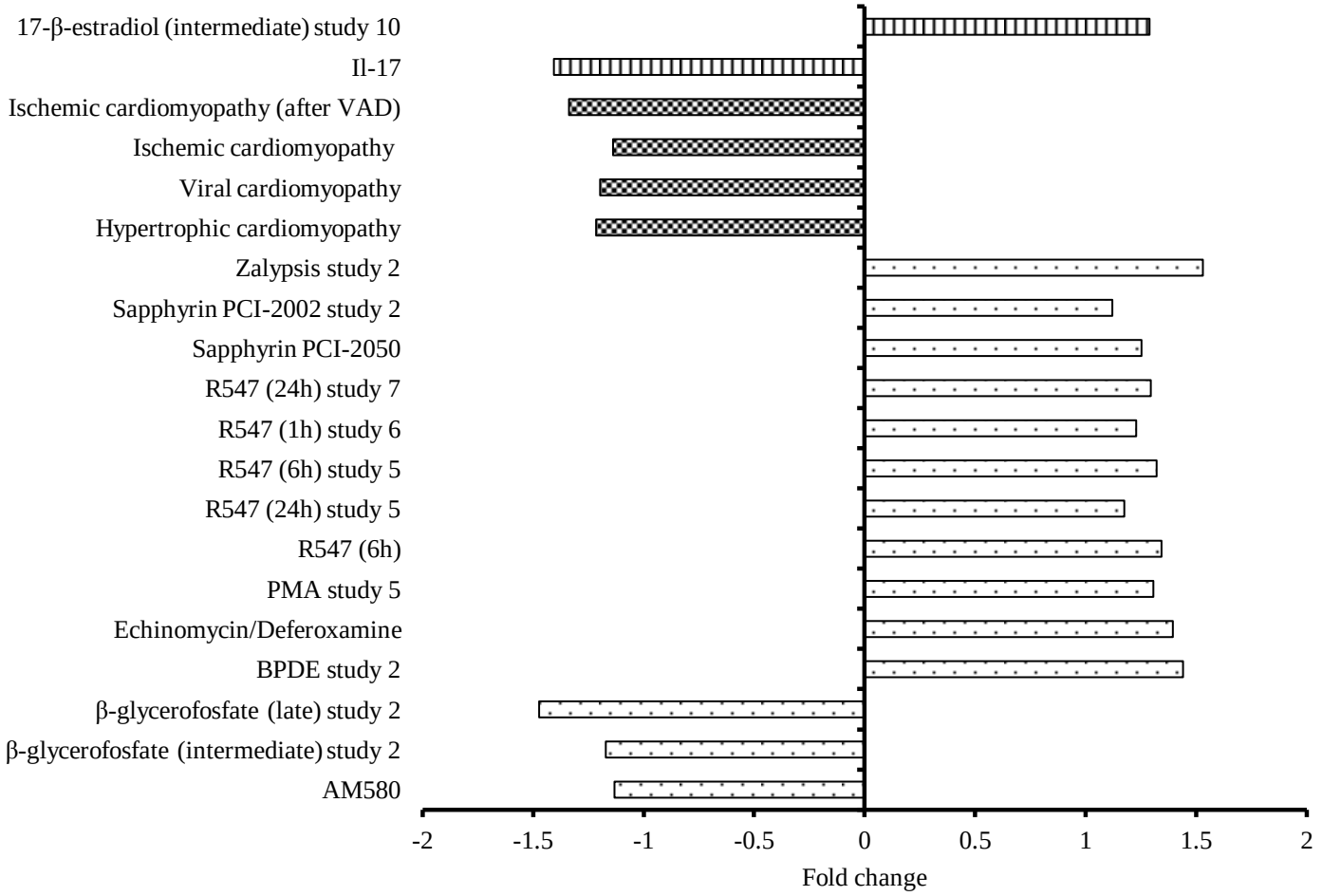
All the chemicals and drugs tested resulted in up-regulation of OAT3. The main up-regulatory effect was observed after treating DU145 (human prostate cancer) cells with the anti-neoplastic R547. Treatment resulted in a 1.38-fold increase in the mRNA levels of OAT3.

OAT1 also responded to a wide range of chemicals, which predominantly triggered up-regulation. The highest up-regulation was observed following treatment of OPM-1 (human myeloma) cells with the anti-myeloma compound zalypsis (1.53-fold change). Down-regulation of OAT1 was only triggered by treatment of bone marrow stromal cells with the phosphatase inhibitor β-glycerophosphate and with the drug AM580, respectively. The former treatment triggered a 1.17-fold change, while the latter induced a 1.13-fold change.

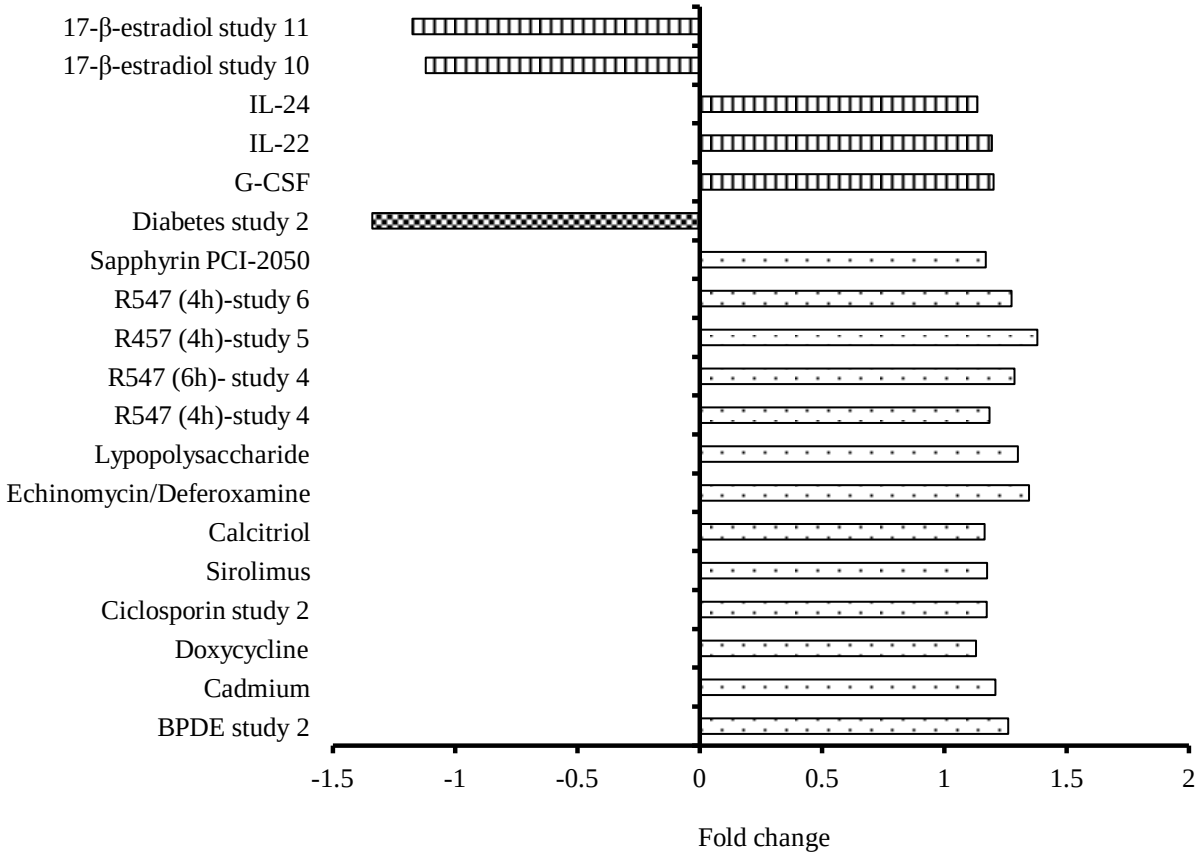
Among the tested biological mediators, OAT3 expression was up-regulated from three cytokines: IL-22, IL-24 (interleukin 22 and 24) and G-CSF (granulocyte colony-stimulating factor). IL-22 and Il-24 were used to treat neonatal epidermal keratinocytes and showed to induce a 1.19- and 1.14-fold change on OAT3, respectively. G-CSF was used on leukocytes and induced a 1.20-fold change on OAT3. OAT1 was down-regulated by IL-17 (1.41-fold decrease) when treating aortic vascular smooth muscle cells. The hormone 17-β-estradiol had opposite effects on OAT3 and OAT1 expressed in MCF-7 (breast cancer) cells, down-regulating OAT3 and up-regulating OAT1 (1.17- and 1.29-fold change, respectively).

All the conditions modulating transcription of OAT1 and OAT3 are shown in figure 3-5.

Figure 3-5. Modulation of the OATs. Modulation is shown for OAT1 (a) and OAT3 (b), using the following filling patterns: dots for chemicals and drugs light vertical for biological mediators, small checker board for disease.


17-β-estradiol (intermediate) study 10
Il-17
Ischemic cardiomyopathy (after VAD)
Ischemic cardiomyopathy
Viral cardiomyopathy
Hypertrophic cardiomyopathy
Zalypsis study 2
Sapphyrin PCI-2002 study 2
Sapphyrin PCI-2050
R547 (24h) study 7
R547 (1h) study 6
R547 (6h) study 5
R547 (24h) study 5
R547 (6h)
PMA study 5
Echinomycin/Deferoxamine
BPDE study 2
β-glycerofosfate (late) study 2
β-glycerofosfate (intermediate) study 2
AM580
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2
Fold change

a.


17-β-estradiol study 11
17-β-estradiol study 10
IL-24
IL-22
G-CSF
Diabetes study 2
Sapphyrin PCI-2050
R547 (4h)-study 6
R457 (4h)-study 5
R547 (6h)- study 4
R547 (4h)-study 4
Lypopolysaccharide
Echinomycin/Deferoxamine
Calcitriol
Sirolimus
Ciclosporin study 2
Doxycycline
Cadmium
BPDE study 2
-1.5
-1
-0.5
0
0.5
1
1.5
2
Fold change

b.

3.4.2.1.2. Modulation of the OATPs (OATP1A2, OATP1B1, OATP1B3 and OATP4C1)

Among the OATPs, OATP1A2 was the least responsive to modulation, responding to 15 conditions. OATP1B1, OATP1B3 and OATP4C1 were modulated from 20, 38 and 30 conditions respectively.

Apple procyanidin and curcumin were the only food compounds tested on the OATPs. Curcumin tested alone induced a 1.12-fold increase on OATP1A2, expressed in U937 (human leukemic monocyte lymphoma) cells. Curcumin, used to treat U937 cells, acted as a down-regulator of OATP1B1, when it was used after pre-treatment with the stressor hydrogen peroxide (H_2O_2). It is noteworthy to mention that pre-treatment with H_2O_2 decreased OATP1B1 expression inducing a 1.23-fold decrease and that subsequent treatment with curcumin reverted this effect, inducing a 1.23-fold increase on OAT1B1.

Among the OATPs, OATP1A2 was the least modulated by chemicals. On the contrary, OATP1B3 was the OATP affected by the largest number of chemicals.

The biggest change triggered from a biological mediator was observed on the expression of OATP1B3. When ASPC-1 (metastatic pancreas cancer) cells were treated with the stromal cell-derived factor 1 (SDF-1) OATP1B3 showed a 4-fold increase.

Multiple drugs showed to affect expression of OATP1B3, mainly up-regulating it. Significant up-regulation was observed on U251 (astroglioma) cells treated with the chelating agent deferoxamine (2.20-fold change). Up-regulation was also triggered form treatment with a range of other chemicals, such as 5-aza-2'deoxycitidine (an inhibitor of DNA methylation) applied to bronchial epithelial cells and R547, used on DU145O (1.47- and 1.42-fold change respectively). On the contrary, OATP4C1 resulted to be predominantly down-regulated from chemical compounds. For example, treatment of monocytes with PMA (phorbol myristate acetate) triggered a 2.5-fold decrease on

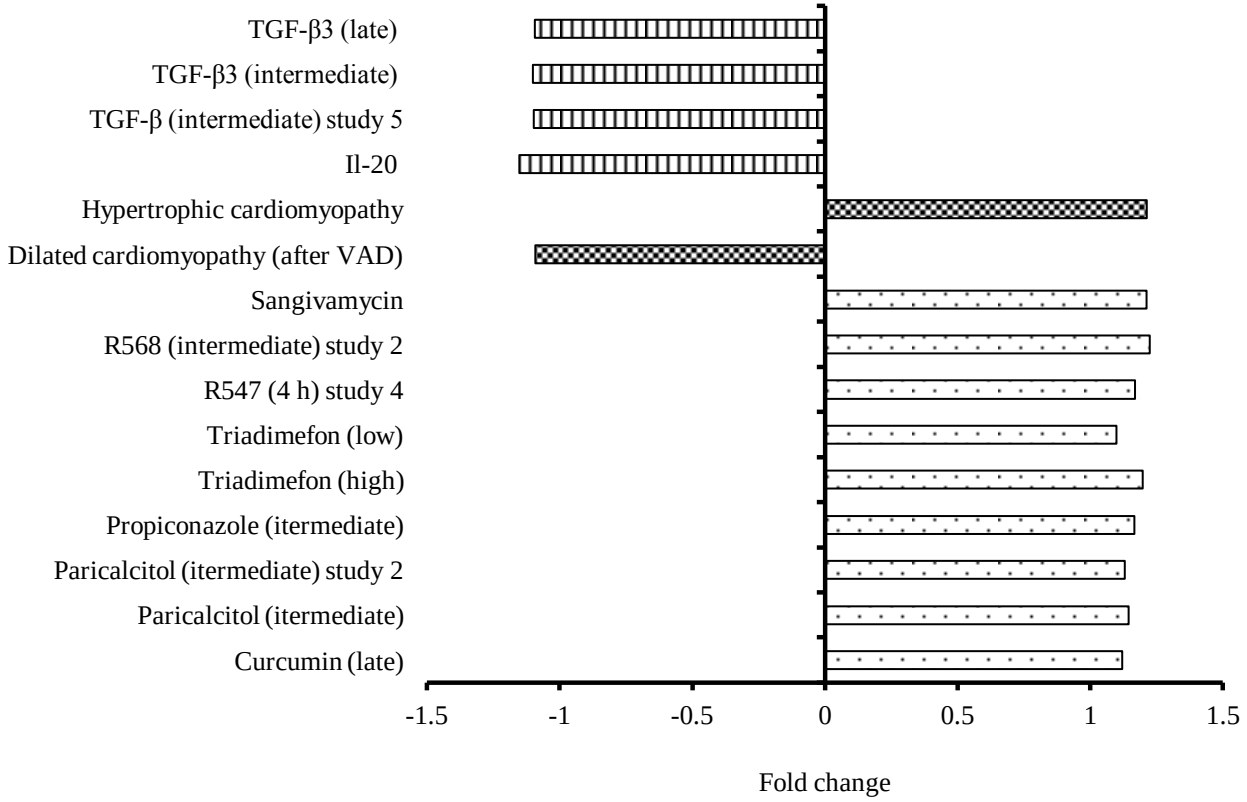
OATP4C1. Among the OATPs, OATP1B3 was the most responsive to modulation by chemicals.

Among the biological mediators, none of the tested hormones affected OATP1A2 or OATP1B1, while OATP4C1 expressed in MCF-7 cells was down-regulated (1.85-fold change) after treatment with 17-β-estradiol. The hormone 17- β-estradiol also down-regulated OAT1B3, expressed in MCF-7 cells, triggering a 1.19 fold change.

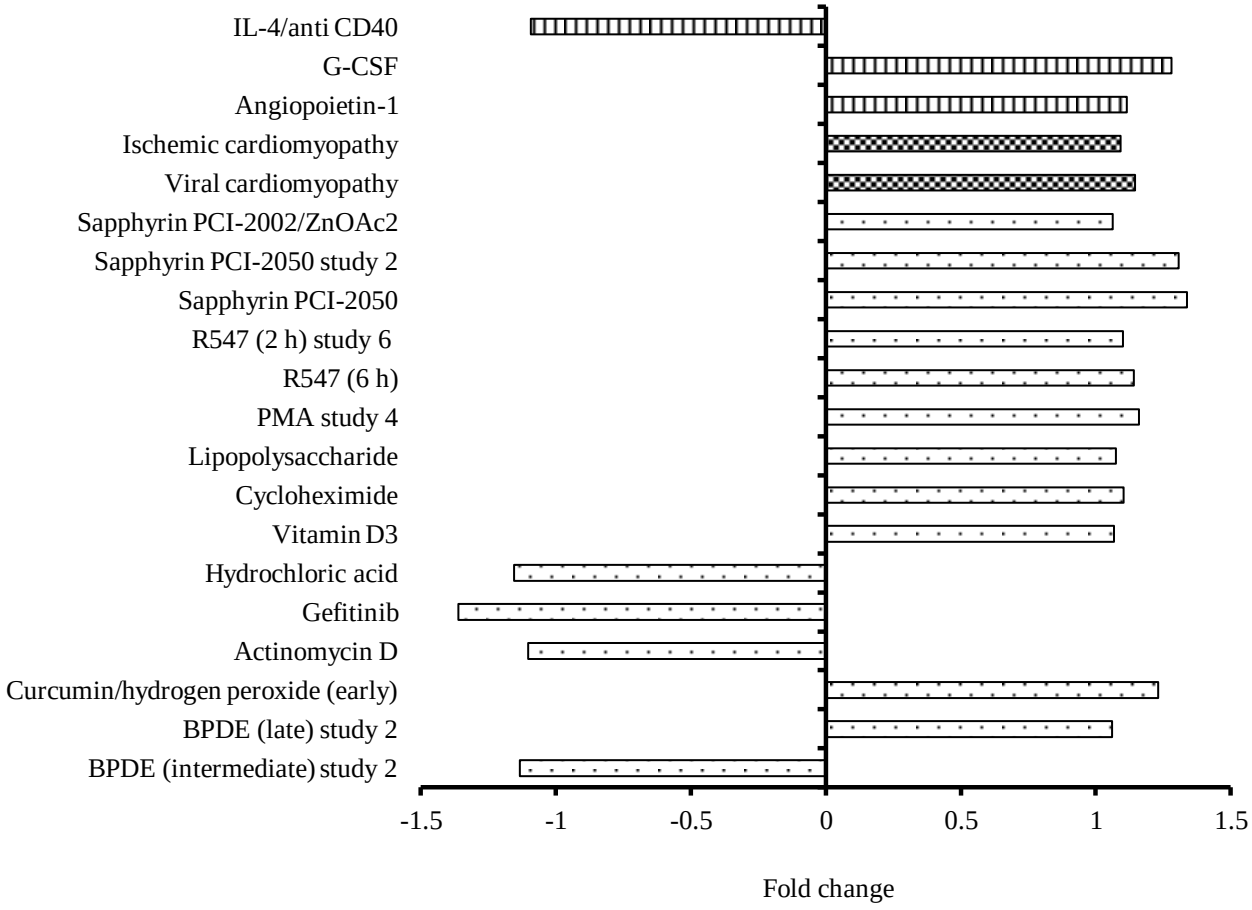
A small range of cytokines and growth factors affected the expression of the OATPs. OATP1A2 underwent a 1.15-fold decrease after treatment with IL-20 in neonatal epidermal keratinocytes. OATP1B1, OATP1B3 and OATP4C1 showed to be mainly down-regulated form the tested cytokines. The main down-regulatory effect was observed on OATP1B3, expressed in metastatic mammary gland cells, following treatment with SDF-1 (4.17-fold decrease). A major effect (2-fold increase) was also observed on OATP4C, after treatment of leukocytes with the G-CSF.

All the conditions modulating transcription of OATP1A2, OATP1B1, OATP1B3 and OATP4C1 are shown in figure 3-6.

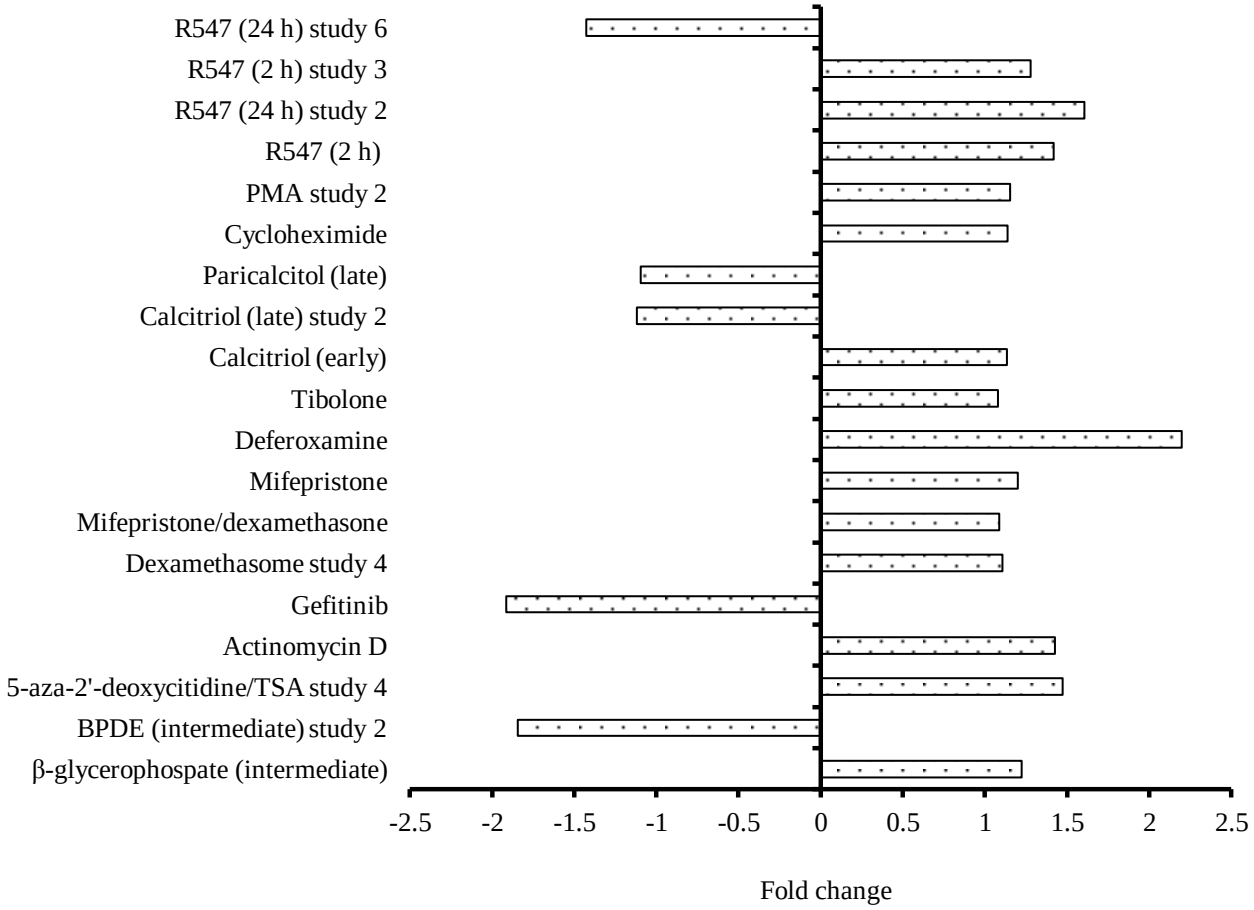
Figure 3-6. Modulation of the OATPs. Modulation is shown for OATP1A2 (a), OATP1B1 (b), OATP1B3 (c) and OATP4C1 (d) using the following filling patterns: dots for chemicals and drugs light vertical for biological mediators, small checker board for disease.


TGF-β3 (late)
TGF-β3 (intermediate)
TGF-β (intermediate) study 5
Il-20
Hypertrophic cardiomyopathy
Dilated cardiomyopathy (after VAD)
Sangivamycin
R568 (intermediate) study 2
R547 (4 h) study 4
Triadimefon (low)
Triadimefon (high)
Propiconazole (itermediate)
Paricalcitol (itermediate) study 2
Paricalcitol (itermediate)
Curcumin (late)
-1.5
-1
-0.5
0
0.5
1
1.5
Fold change

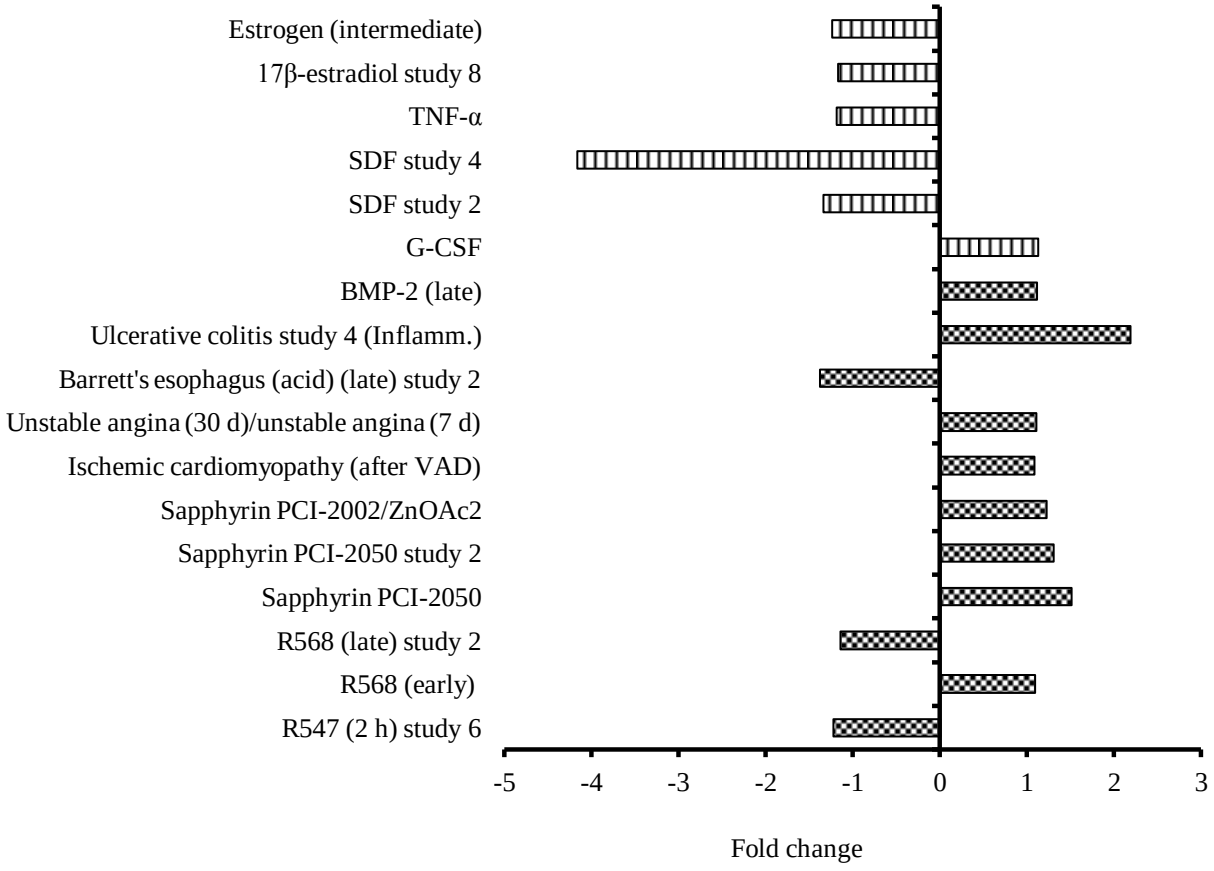
a.


IL-4/anti CD40
G-CSF
Angiopoietin-1
Ischemic cardiomyopathy
Viral cardiomyopathy
Sapphyrin PCI-2002/ZnOAc2
Sapphyrin PCI-2050 study 2
Sapphyrin PCI-2050
R547 (2 h) study 6
R547 (6 h)
PMA study 4
Lipopolysaccharide
Cycloheximide
Vitamin D3
Hydrochloric acid
Gefitinib
Actinomycin D
Curcumin/hydrogen peroxide (early)
BPDE (late) study 2
BPDE (intermediate) study 2
-1.5
-1
-0.5
0
0.5
1
1.5
Fold change

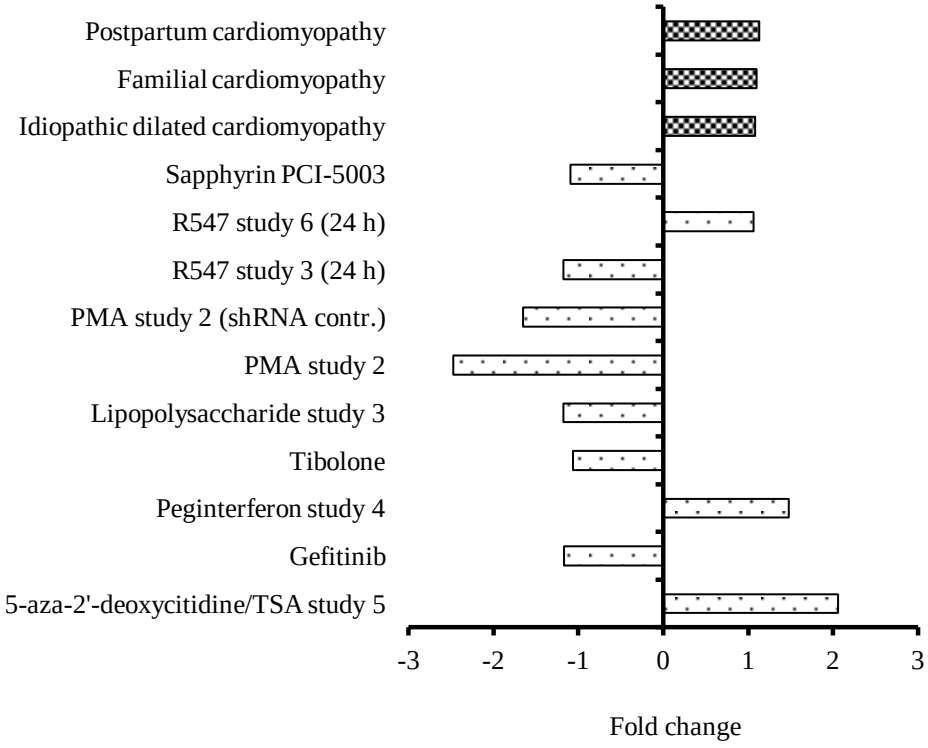
b.


R547 (24 h) study 6
R547 (2 h) study 3
R547 (24 h) study 2
R547 (2 h)
PMA study 2
Cycloheximide
Paricalcitol (late)
Calcitriol (late) study 2
Calcitriol (early)
Tibolone
Deferoxamine
Mifepristone
Mifepristone/dexamethasone
Dexamethasome study 4
Gefitinib
Actinomycin D
5-aza-2'-deoxycitidine/TSA study 4
BPDE (intermediate) study 2
β-glycerophospate (intermediate)
-2.5
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2
2.5
Fold change

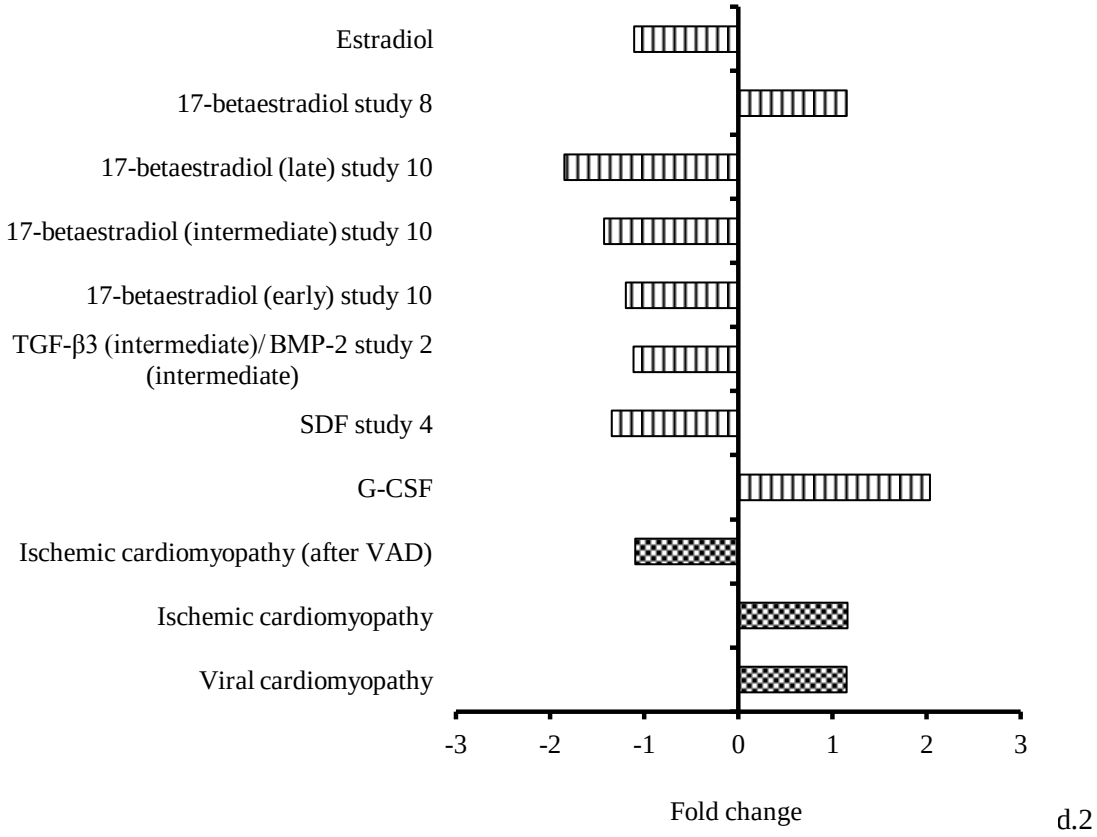
c.1


Estrogen (intermediate)
17β-estradiol study 8
TNF-α
SDF study 4
SDF study 2
G-CSF
BMP-2 (late)
Ulcerative colitis study 4 (Inflamm.)
Barrett's esophagus (acid) (late) study 2
Unstable angina (30 d)/unstable angina (7 d)
Ischemic cardiomyopathy (after VAD)
Sapphyrin PCI-2002/ZnOAc2
Sapphyrin PCI-2050 study 2
Sapphyrin PCI-2050
R568 (late) study 2
R568 (early)
R547 (2 h) study 6
-5
-4
-3
-2
-1
0
1
2
3
Fold change

c.2


Postpartum cardiomyopathy
Familial cardiomyopathy
Idiopathic dilated cardiomyopathy
Sapphyrin PCI-5003
R547 study 6 (24 h)
R547 study 3 (24 h)
PMA study 2 (shRNA contr.)
PMA study 2
Lipopolysaccharide study 3
Tibolone
Peginterferon study 4
Gefitinib
5-aza-2'-deoxycitidine/TSA study 5
-3
-2
-1
0
1
2
3
Fold change

d.1


Estradiol
17-betaestradiol study 8
17-betaestradiol (late) study 10
17-betaestradiol (intermediate) study 10
17-betaestradiol (early) study 10
TGF-β3 (intermediate)/ BMP-2 study 2 (intermediate)
SDF study 4
G-CSF
Ischemic cardiomyopathy (after VAD)
Ischemic cardiomyopathy
Viral cardiomyopathy
-3
-2
-1
0
1
2
3
Fold change

d.2

3.4.2.1.3. Modulation of the ABCs (MRP2, MRP3 and BCRP)

Among the ABCs, MRP3 and BCRP were the least and the most modulated transporter, respectively. The total number of conditions affecting expression was 37 for MRP2, 35 for MRP3 and 54 for BCRP.

As for the other transporters discussed so far, apple procyanidin and curcumin were the only food compounds tested. Neither apple procyanidins nor curcumin had any effect on MRP2 or MRP3 (data not shown). BCRP showed a 1.23-fold decrease when HUVEC (human umbilical vein endothelial) cells were treated with apple procyanidin, applied to cells following pre-incubation with the stressor TNF-α (tumor necrosis factor-α). Pre-incubation with TNF- α up-regulated BCRP (1.23-fold increase), subsequently incubation with apple procyanidins reverted the effect of the tumor necrosis factor. It is noteworthy that treatment of HUVEC cells with apple procyanidins alone (without pre-incubation with TNF- α) triggered no change in the mRNA levels of BCRP.

Multiple chemical compounds affected the expression of the ABC transporters. A significant effect (64-fold increase) was observed on BCRP when treating ASPC-1 cells with PMA. PMA also up-regulated MRP2 in ASPC-1 cells (5-fold change).

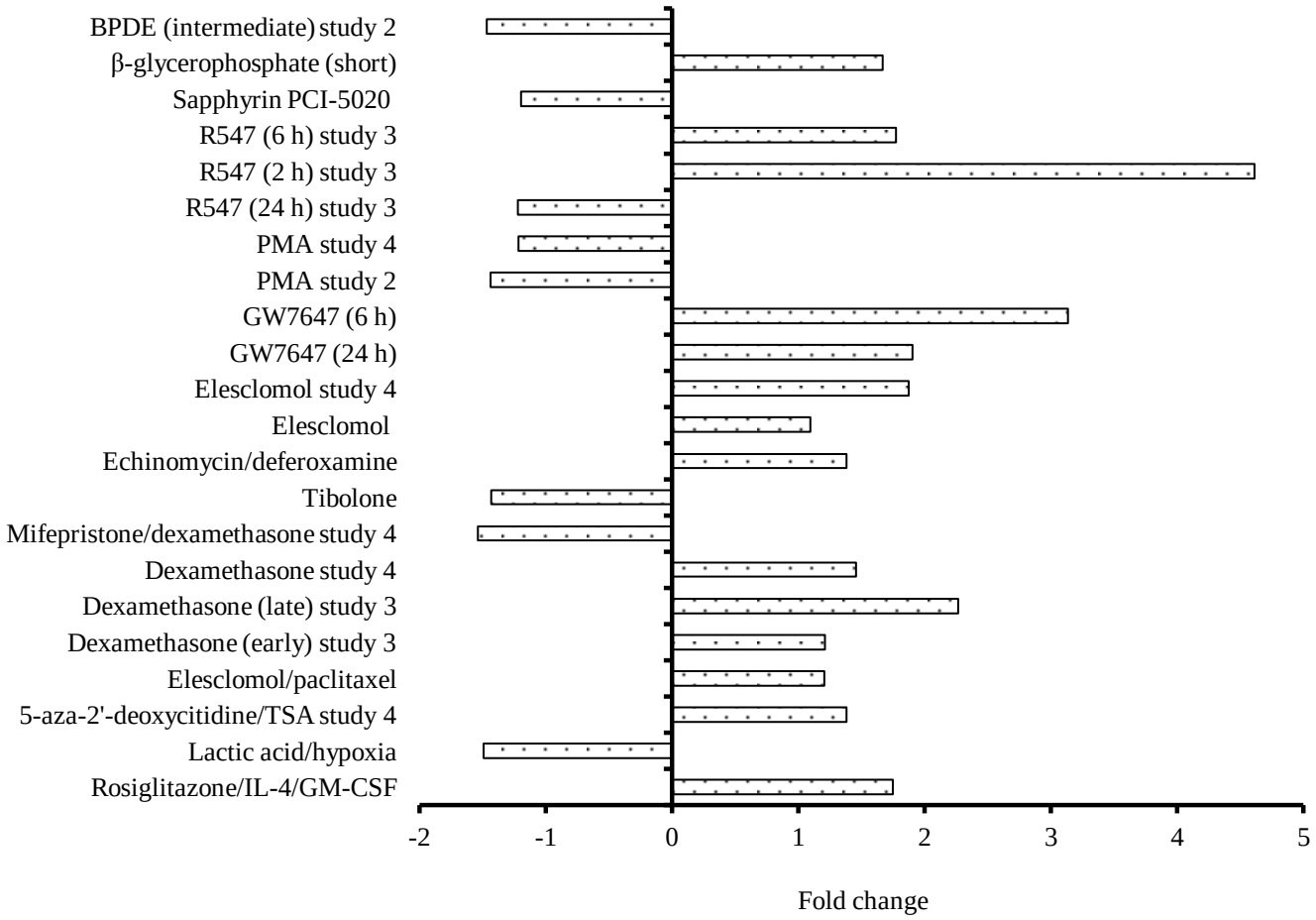
On the contrary, PMA slightly down-regulated MRP3 in monocytic cells, triggering a 1.04-fold change. The main up-regulatory effect on BCRP was observed when treating MCF-7 cells with the anti-diabetic drug rosiglitazone (7-fold increase), while the biggest response triggered on MRP2 (3.13-fold increase) was observed after treatment of melanoma cells with the apoptosis-inducer elesclomol.

The transcriptional levels of MRP2 were not affected from any of the tested hormones, while MRP3 was down-regulated by all the tested hormones. Transcription of BCRP was altered the most after treatment with 17 –β-estradiol, resulting in a 2.15-fold increase when MCF-7 cells were treated.

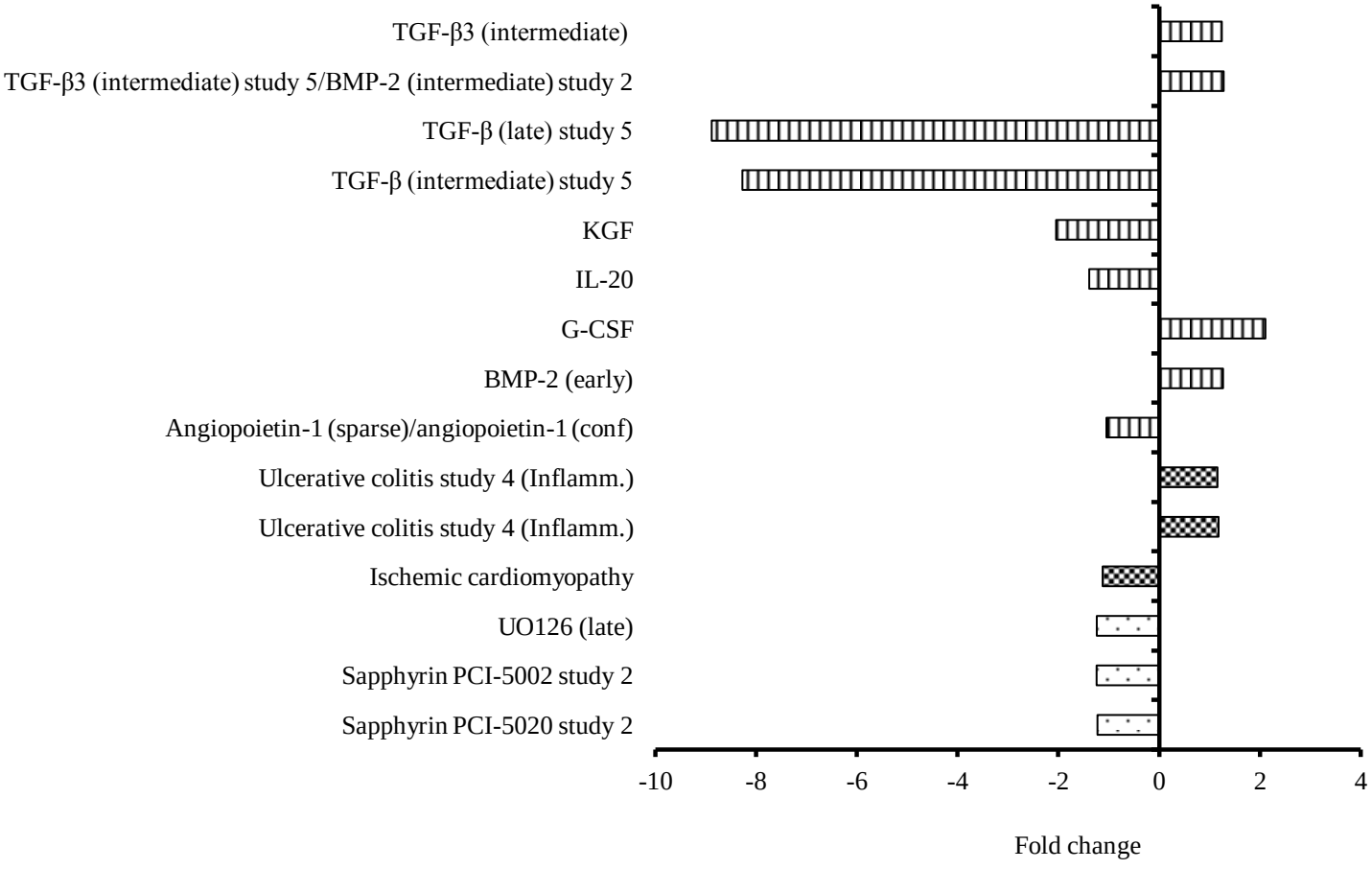
A range of cytokines and growth factors affected the expression of MRP2, MRP3 and BCRP. An example is the TGF- β (transforming growth factor β), that triggered a 9- and a 13-fold increase in MRP2 and BCR, respectively, in A549 (adenocarcinomic human alveolar basal epithelial) cells. Overall MRP2 was the ABC affected by the largest number of cytokines and growth factors, with an inconsistent pattern of modulation. MRP3 and BCRP mainly resulted in up- and down-regulation, respectively, when exposed to growth factors and cytokines.

All the conditions modulating the transcription of MRP2, MRP3 and BCRP are shown in figure 3-7.

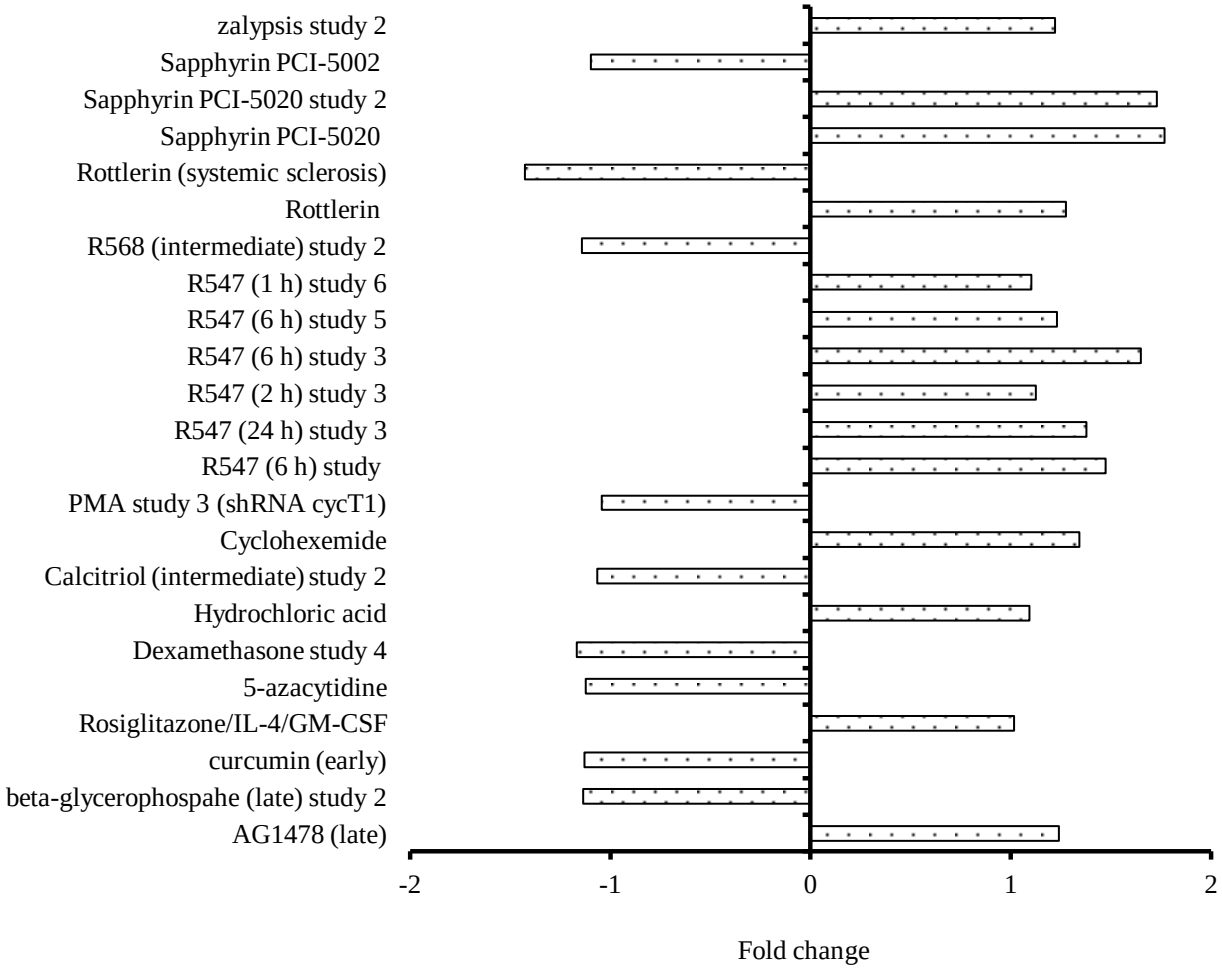
Figure 3-7. Modulation of ABCs. Modulation is shown for MRP2 (a) MRP3 (b) and BCRP (c), using the following filling patterns: dots for chemicals and drugs light vertical for biological mediators, small checker board for disease.


BPDE (intermediate) study 2
β-glycerophosphate (short)
Sapphyrin PCI-5020
R547 (6 h) study 3
R547 (2 h) study 3
R547 (24 h) study 3
PMA study 4
PMA study 2
GW7647 (6 h)
GW7647 (24 h)
Elesclomol study 4
Elesclomol
Echinomycin/deferoxamine
Tibolone
Mifepristone/dexamethasone study 4
Dexamethasone study 4
Dexamethasone (late) study 3
Dexamethasone (early) study 3
Elesclomol/paclitaxel
5-aza-2'-deoxycitidine/TSA study 4
Lactic acid/hypoxia
Rosiglitazone/IL-4/GM-CSF
-2
-1
0
1
2
3
4
5
Fold change

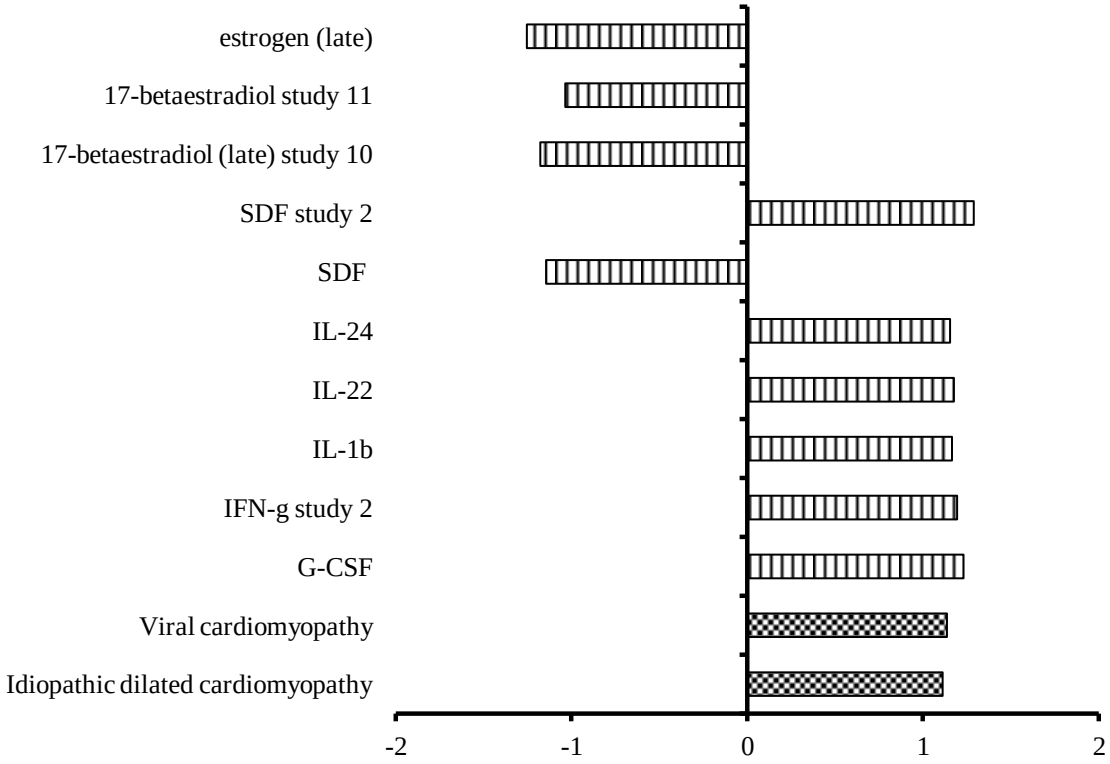
a.1


TGF-β3 (intermediate)
TGF-β3 (intermediate) study 5/BMP-2 (intermediate) study 2
TGF-β (late) study 5
TGF-β (intermediate) study 5
KGF
IL-20
G-CSF
BMP-2 (early)
Angiopoietin-1 (sparse)/angiopoietin-1 (conf)
Ulcerative colitis study 4 (Inflamm.)
Ulcerative colitis study 4 (Inflamm.)
Ischemic cardiomyopathy
UO126 (late)
Sapphyrin PCI-5002 study 2
Sapphyrin PCI-5020 study 2
-10
-8
-6
-4
-2
0
2
4
Fold change

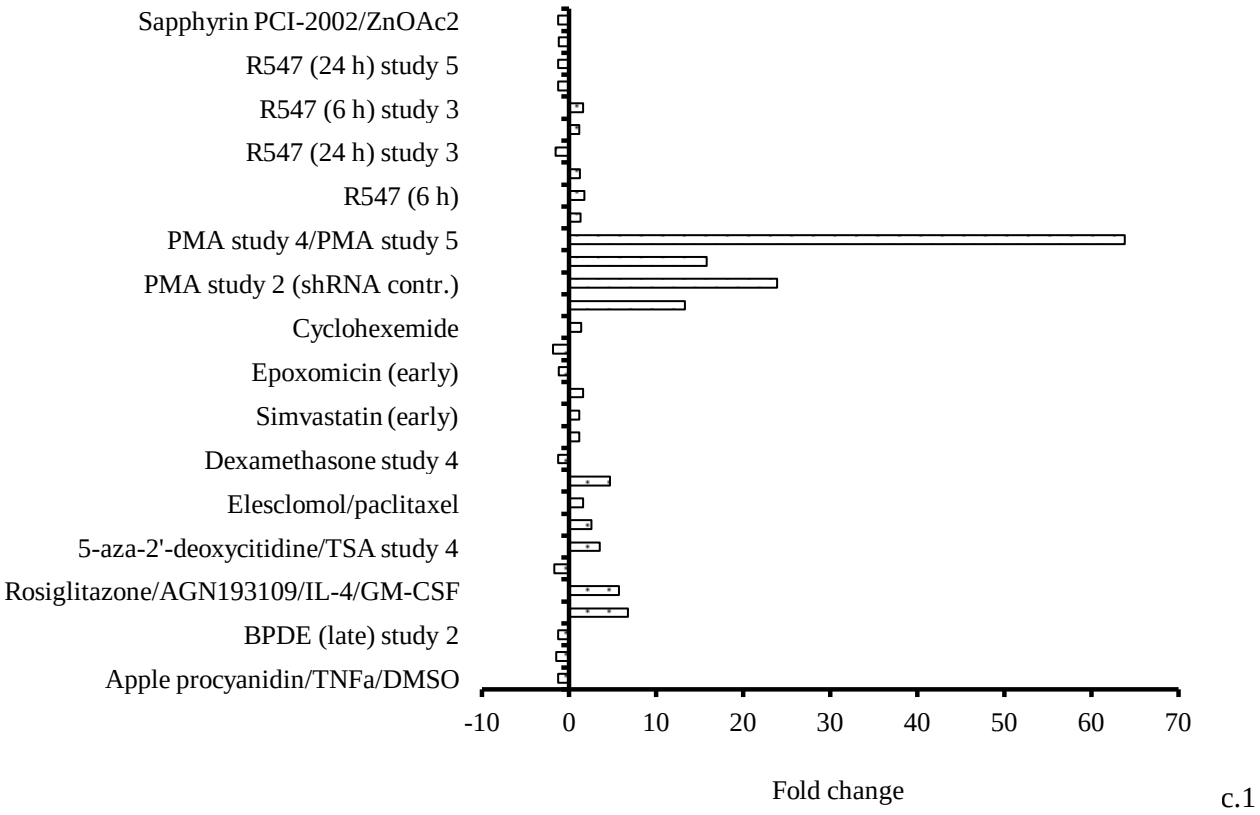
a.2


zalypsis study 2
Sapphyrin PCI-5002
Sapphyrin PCI-5020 study 2
Sapphyrin PCI-5020
Rottlerin (systemic sclerosis)
Rottlerin
R568 (intermediate) study 2
R547 (1 h) study 6
R547 (6 h) study 5
R547 (6 h) study 3
R547 (2 h) study 3
R547 (24 h) study 3
R547 (6 h) study
PMA study 3 (shRNA cycT1)
Cyclohexemide
Calcitriol (intermediate) study 2
Hydrochloric acid
Dexamethasone study 4
5-azacytidine
Rosiglitazone/IL-4/GM-CSF
curcumin (early)
beta-glycerophospahe (late) study 2
AG1478 (late)
-2
-1
0
1
2
Fold change

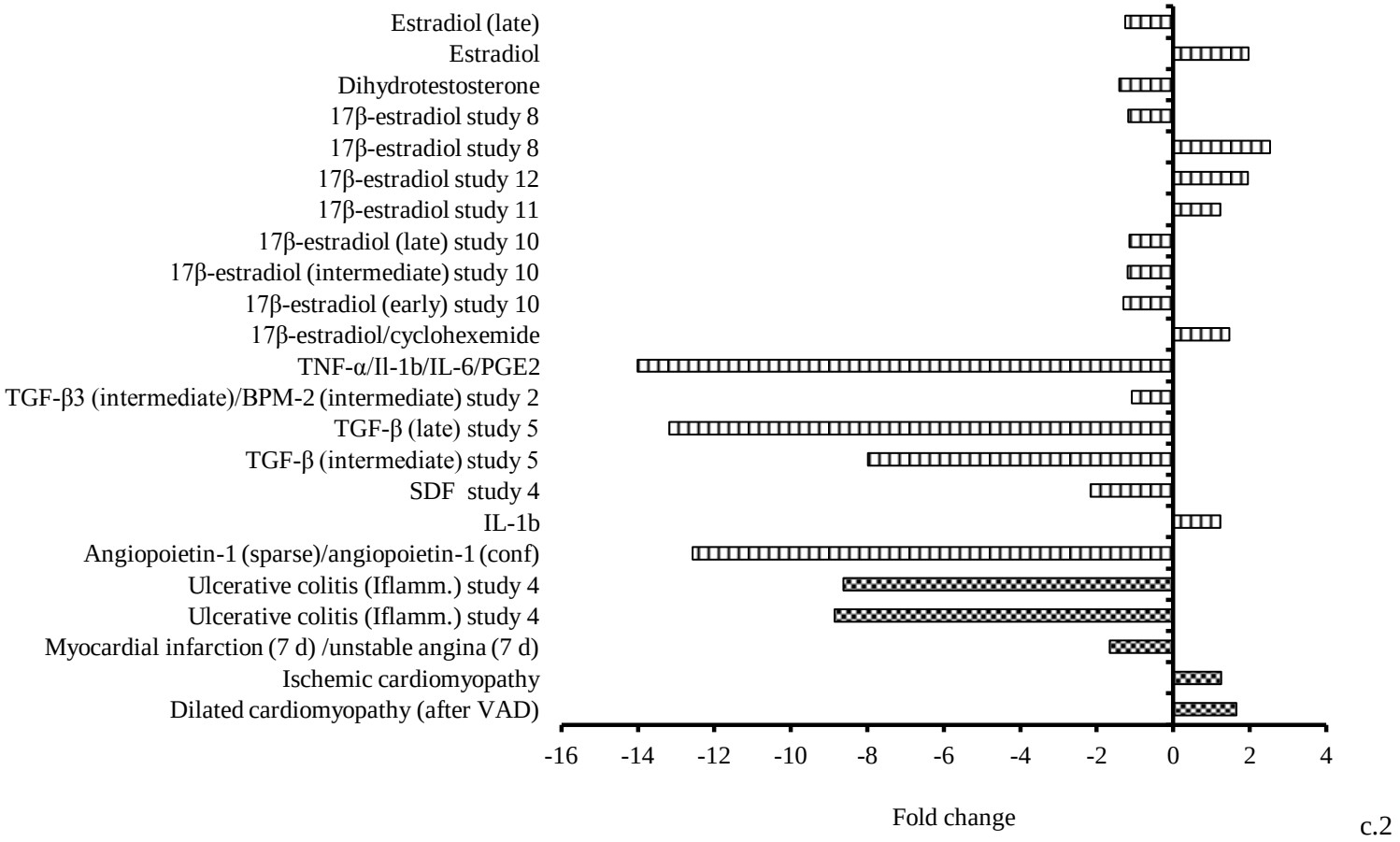
b.1


estrogen (late)
17-betaestradiol study 11
17-betaestradiol (late) study 10
SDF study 2
SDF
IL-24
IL-22
IL-1b
IFN-g study 2
G-CSF
Viral cardiomyopathy
Idiopathic dilated cardiomyopathy
-2
-1
0
1
2
Fold change

b.2


Sapphyrin PCI-2002/ZnOAc2
R547 (24 h) study 5
R547 (6 h) study 3
R547 (24 h) study 3
R547 (6 h)
PMA study 4/PMA study 5
PMA study 2 (shRNA contr.)
Cyclohexemide
Epoxomicin (early)
Simvastatin (early)
Dexamethasone study 4
Elesclomol/paclitaxel
5-aza-2'-deoxycitidine/TSA study 4
Rosiglitazone/AGN193109/IL-4/GM-CSF
BPDE (late) study 2
Apple procyanidin/TNFa/DMSO
-10
0
10
20
30
40
50
60
70
Fold change

c.1


Estradiol (late)
Estradiol
Dihydrotestosterone
17β-estradiol study 8
17β-estradiol study 8
17β-estradiol study 12
17β-estradiol study 11
17β-estradiol (late) study 10
17β-estradiol (intermediate) study 10
17β-estradiol (early) study 10
17β-estradiol/cyclohexemide
TNF-α/Il-1b/IL-6/PGE2
TGF-β3 (intermediate)/BPM-2 (intermediate) study 2
TGF-β (late) study 5
TGF-β (intermediate) study 5
SDF study 4
IL-1b
Angiopoietin-1 (sparse)/angiopoietin-1 (conf)
Ulcerative colitis (Iflamm.) study 4
Ulcerative colitis (Iflamm.) study 4
Myocardial infarction (7 d) /unstable angina (7 d)
Ischemic cardiomyopathy
Dilated cardiomyopathy (after VAD)
-16
-14
-12
-10
-8
-6
-4
-2
0
2
4
Fold change

c.2

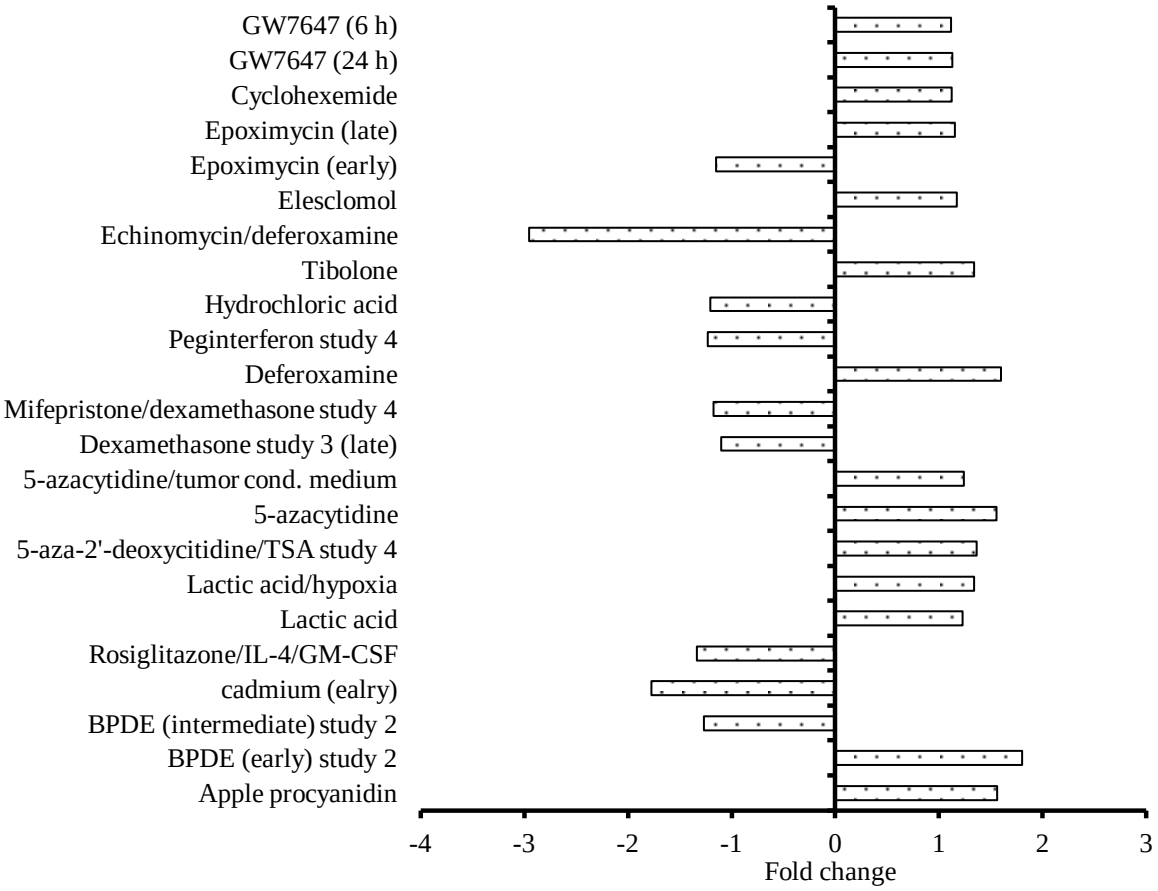
3.4.2.1.4. Modulation of the MCTs (MCT1, MCT7 and SMCT1)

Among this group of transporters, MCT1 and SMCT1 showed to be the members responding to the largest and smallest number of conditions, respectively (61 and 25). Of the tested food compounds, apple procyanidin tested alone triggered a 1.56-fold increase on MCT1, expressed in HUVEC cells. Apple procyanidins affected the transcription of SMCT1in lymphatic cells, only when pre-treatment with TNF-α had been carried out. Treatment with the tumor necrosis factor resulted in a 1.18-fold decrease of the SMCT1 mRNA levels and subsequent treatment with apple procyanidins reversed this effect, by triggering a 1.18-fold increase on SMCT1.

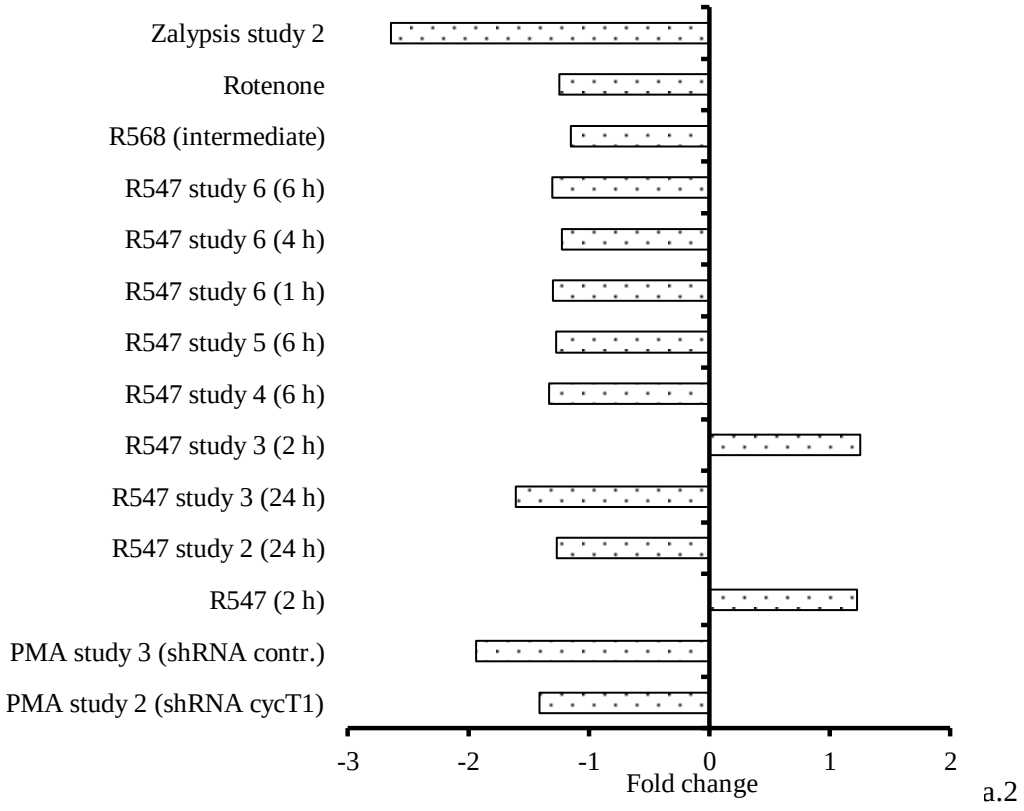
A wide range of chemicals affected the mRNA levels of the MCTs. MCT1 resulted predominantly down-regulated, while MCT7 and SMCT1 were predominantly up-regulated. Of the tested compounds, the major effect was observed on MCT1 and MCT7 expressed in astroglioma cells, after co-incubation with the drugs deferoxamine and echinomycin. A 2.95-fold decrease was observed for MCT1, while a 2.29-fold increase was observed for MCT7. Both MCT1 and MCT7, expressed in ASPC-1 cells, were down-regulated by PMA (1.94- and 1.52-fold decrease, respectively).

A wide range of biological mediators modulated the transcription of MCT1 and MCT7, while a limited number of biological mediators affected SMCT1. MCT1 and SMCT1 were mainly up-regulated from the tested biological mediators, while MCT7 was down-regulated from all the tested hormones. The major effect was observed on MCT7, expressed in A549 cells. When cells were treated with TGF-β a 14.63-fold increase in the mRNA levels of MCT7 was observed. All the conditions modulating the transcription of MRP2, MRP3 and BCRP are shown in figure 3-8.

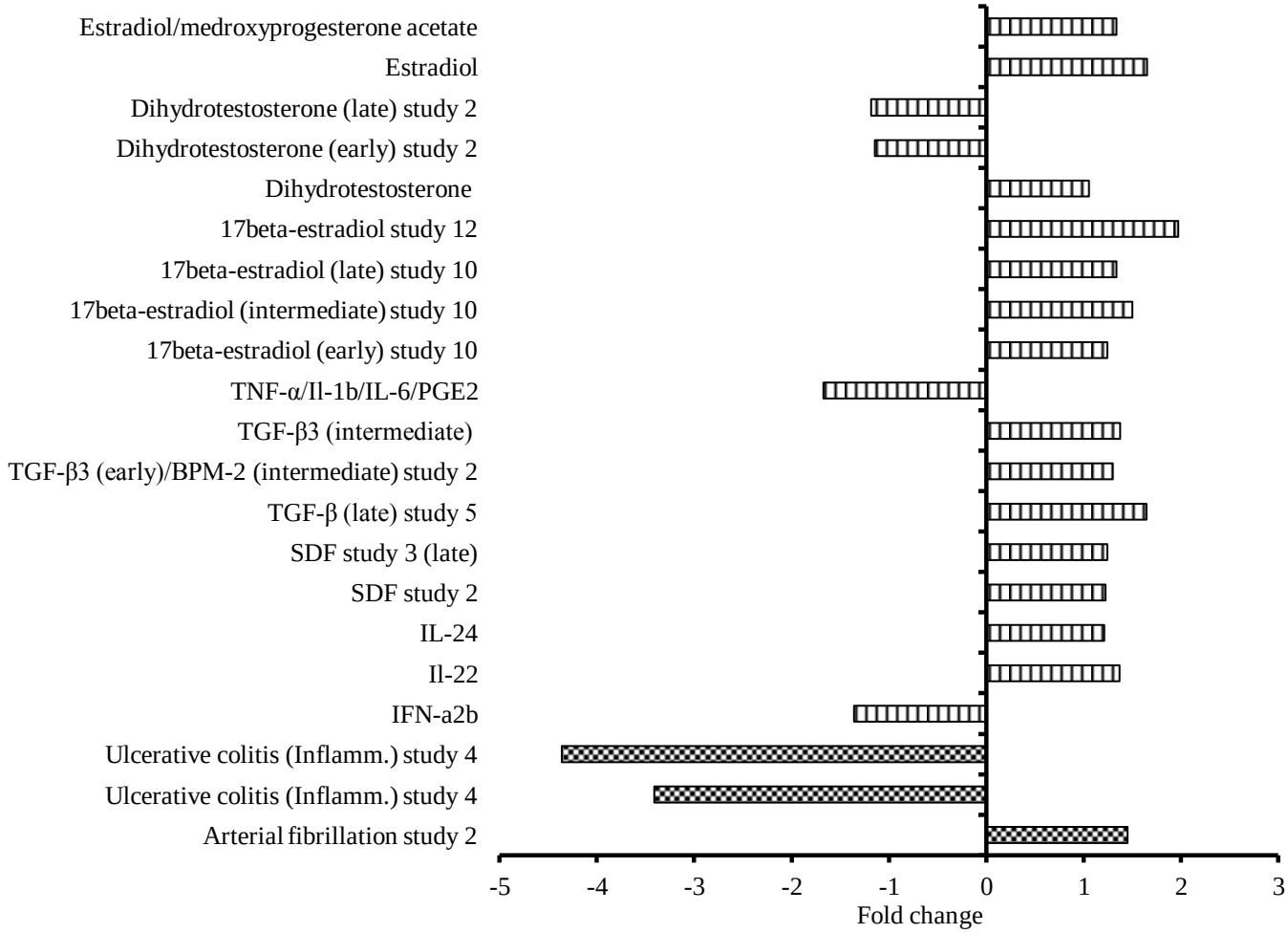
Figure 3-8. Modulation of the MCTs. Modulation is shown for MCT1 (a) MCT7 (b) and SMCT1 (c), using the following filling patterns: dots for chemicals and drugs light vertical for biological mediators, small checker board for disease.


GW7647 (6 h)
GW7647 (24 h)
Cyclohexemide
Epoximycin (late)
Epoximycin (early)
Elesclomol
Echinomycin/deferoxamine
Tibolone
Hydrochloric acid
Peginterferon study 4
Deferoxamine
Mifepristone/dexamethasone study 4
Dexamethasone study 3 (late)
5-azacytidine/tumor cond. medium
5-azacytidine
5-aza-2'-deoxycitidine/TSA study 4
Lactic acid/hypoxia
Lactic acid
Rosiglitazone/IL-4/GM-CSF
cadmium (ealry)
BPDE (intermediate) study 2
BPDE (early) study 2
Apple procyanidin
-4
-3
-2
-1
0
1
2
3
Fold change

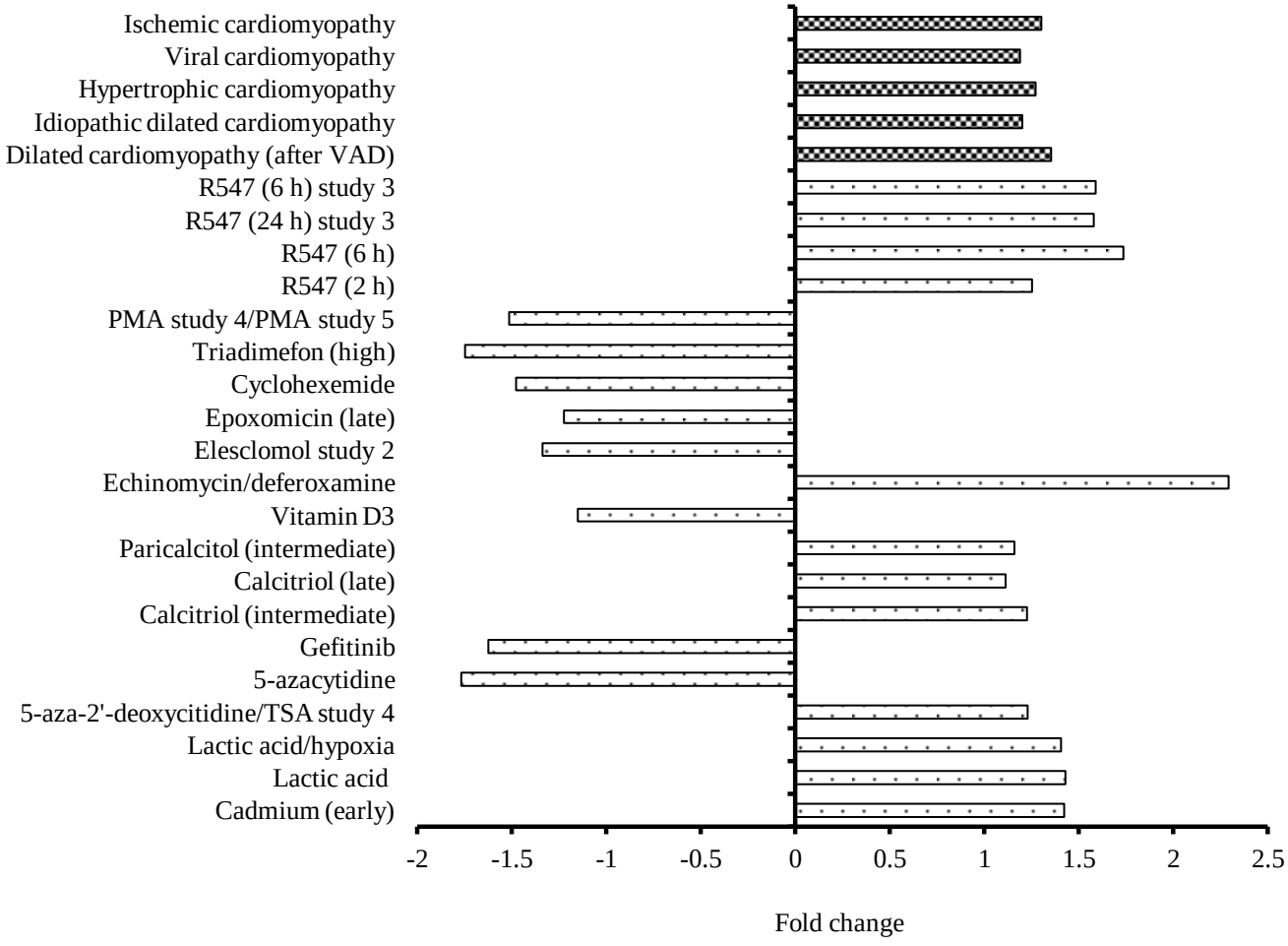
a.1


Zalypsis study 2
Rotenone
R568 (intermediate)
R547 study 6 (6 h)
R547 study 6 (4 h)
R547 study 6 (1 h)
R547 study 5 (6 h)
R547 study 4 (6 h)
R547 study 3 (2 h)
R547 study 3 (24 h)
R547 study 2 (24 h)
R547 (2 h)
PMA study 3 (shRNA contr.)
PMA study 2 (shRNA cycT1)
-3
-2
-1
0
1
2
Fold change

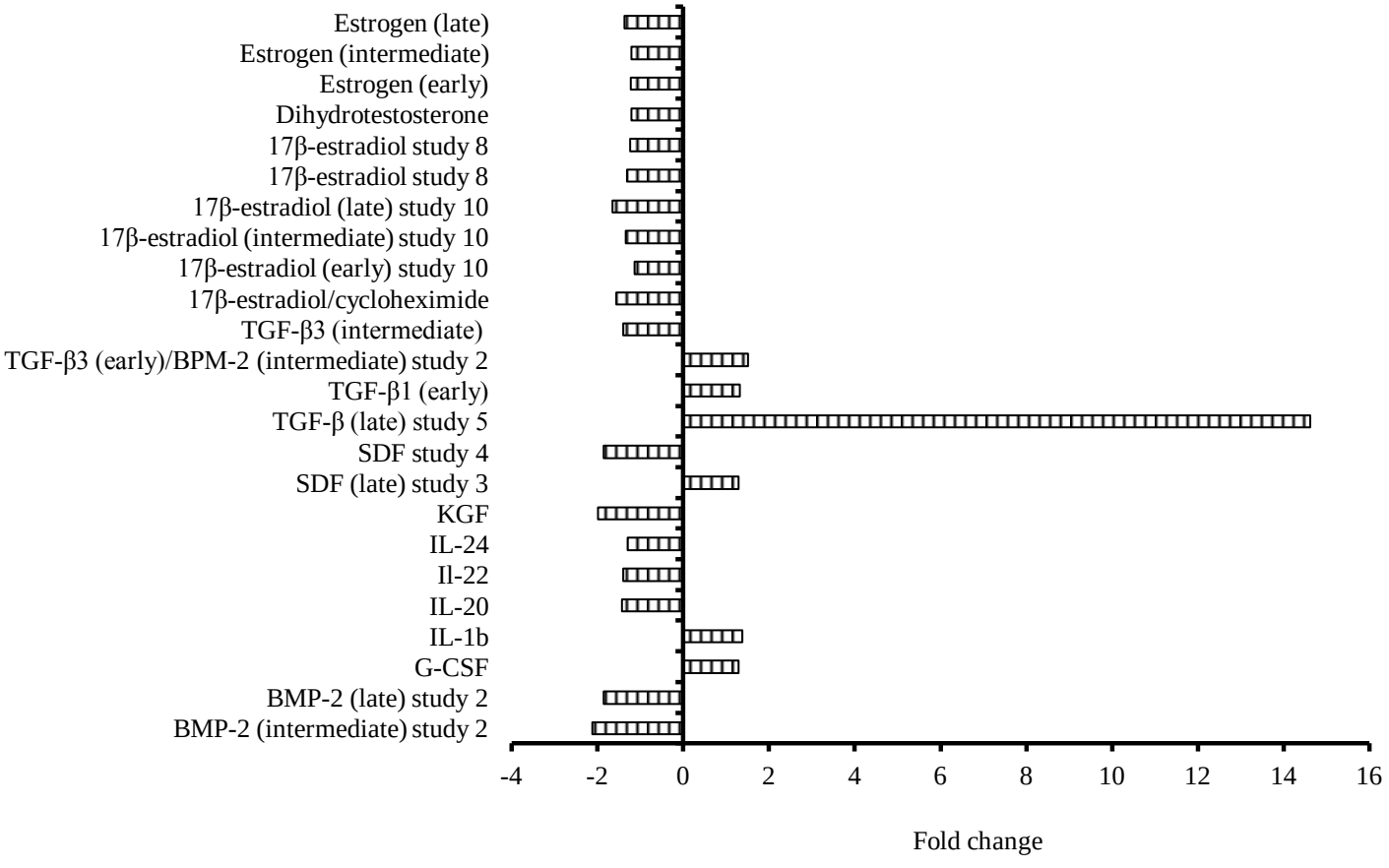
a.2


Estradiol/medroxyprogesterone acetate
Estradiol
Dihydrotestosterone (late) study 2
Dihydrotestosterone (early) study 2
Dihydrotestosterone
17beta-estradiol study 12
17beta-estradiol (late) study 10
17beta-estradiol (intermediate) study 10
17beta-estradiol (early) study 10
TNF-α/Il-1b/IL-6/PGE2
TGF-β3 (intermediate)
TGF-β3 (early)/BPM-2 (intermediate) study 2
TGF-β (late) study 5
SDF study 3 (late)
SDF study 2
IL-24
Il-22
IFN-a2b
Ulcerative colitis (Inflamm.) study 4
Ulcerative colitis (Inflamm.) study 4
Arterial fibrillation study 2
-5
-4
-3
-2
-1
0
1
2
3
Fold change

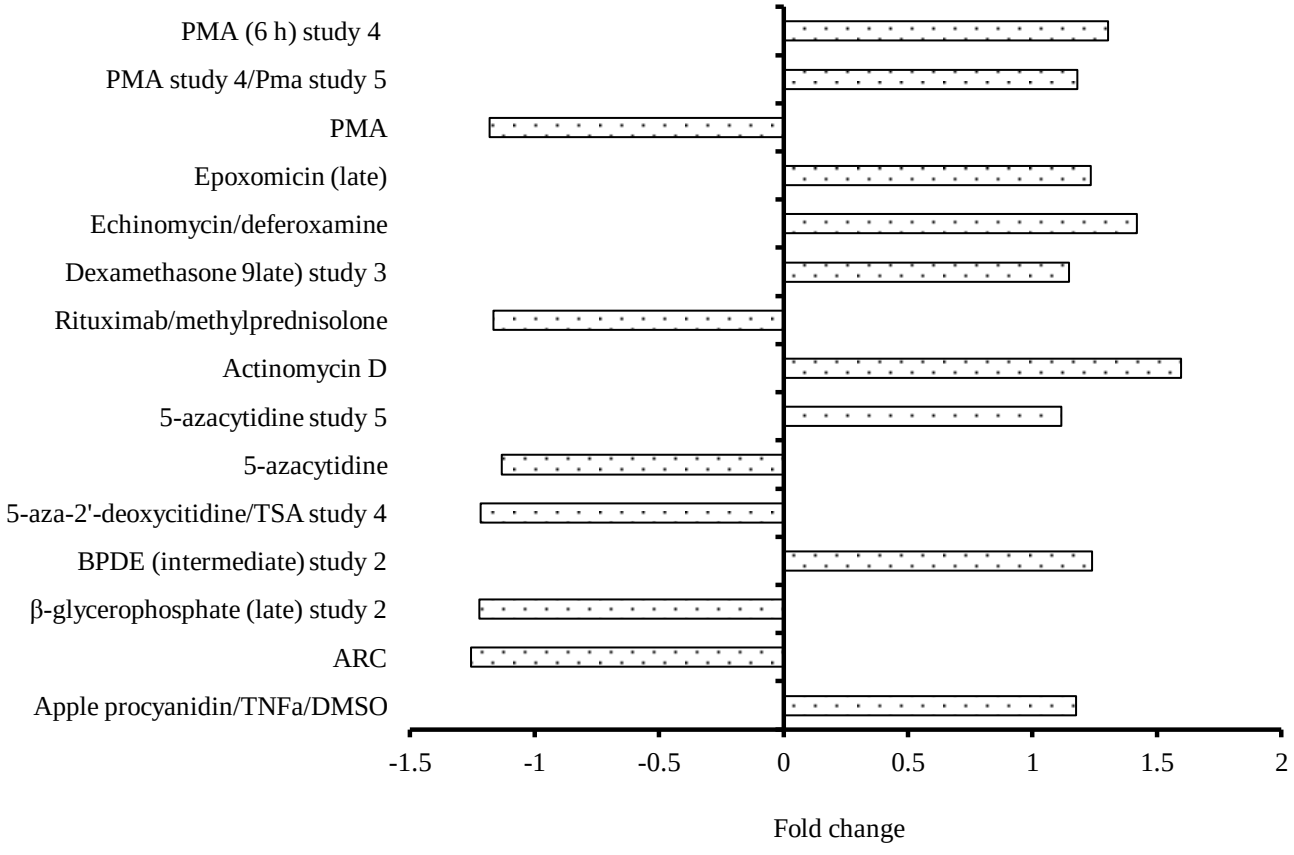
a.3


Ischemic cardiomyopathy
Viral cardiomyopathy
Hypertrophic cardiomyopathy
Idiopathic dilated cardiomyopathy
Dilated cardiomyopathy (after VAD)
R547 (6 h) study 3
R547 (24 h) study 3
R547 (6 h)
R547 (2 h)
PMA study 4/PMA study 5
Triadimefon (high)
Cyclohexemide
Epoxomicin (late)
Elesclomol study 2
Echinomycin/deferoxamine
Vitamin D3
Paricalcitol (intermediate)
Calcitriol (late)
Calcitriol (intermediate)
Gefitinib
5-azacytidine
5-aza-2'-deoxycitidine/TSA study 4
Lactic acid/hypoxia
Lactic acid
Cadmium (early)
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2
2.5
Fold change

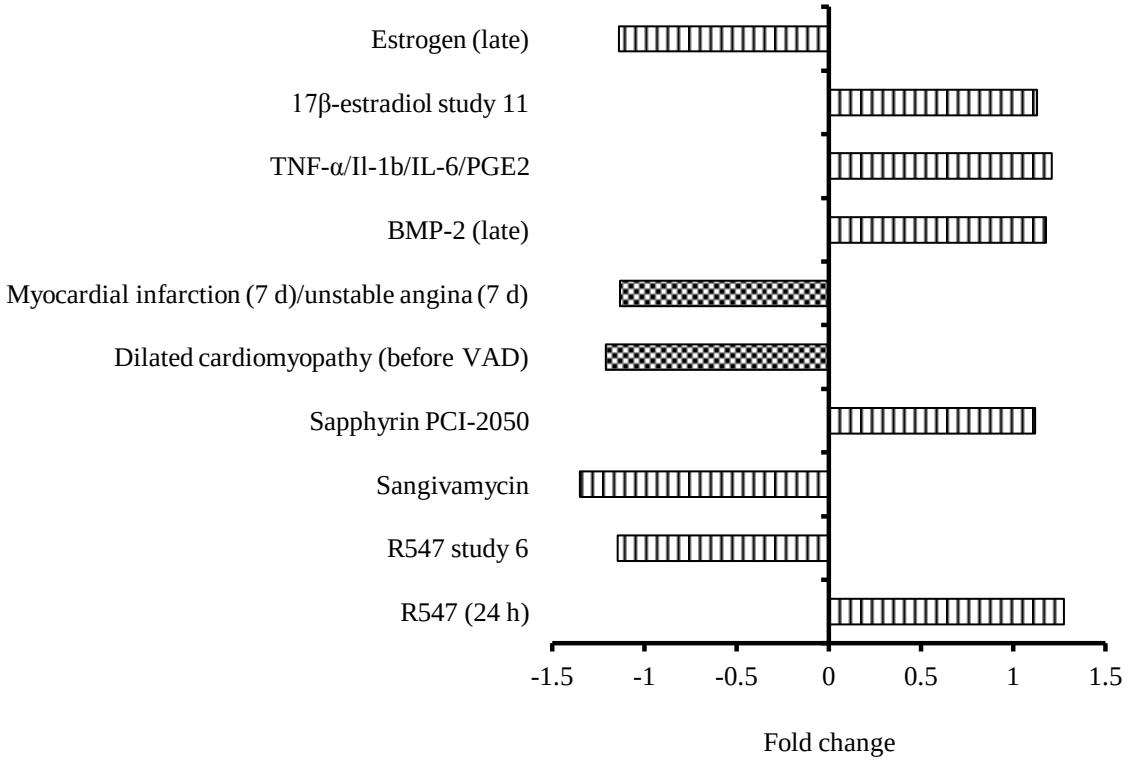
b.1


Estrogen (late)
Estrogen (intermediate)
Estrogen (early)
Dihydrotestosterone
17β-estradiol study 8
17β-estradiol study 8
17β-estradiol (late) study 10
17β-estradiol (intermediate) study 10
17β-estradiol (early) study 10
17β-estradiol/cycloheximide
TGF-β3 (intermediate)
TGF-β3 (early)/BPM-2 (intermediate) study 2
TGF-β1 (early)
TGF-β (late) study 5
SDF study 4
SDF (late) study 3
KGF
IL-24
Il-22
IL-20
IL-1b
G-CSF
BMP-2 (late) study 2
BMP-2 (intermediate) study 2
-4
-2
0
2
4
6
8
10
12
14
16
Fold change

b.2


PMA (6 h) study 4
PMA study 4/Pma study 5
PMA
Epoxomicin (late)
Echinomycin/deferoxamine
Dexamethasone 9late) study 3
Rituximab/methylprednisolone
Actinomycin D
5-azacytidine study 5
5-azacytidine
5-aza-2'-deoxycitidine/TSA study 4
BPDE (intermediate) study 2
β-glycerophosphate (late) study 2
ARC
Apple procyanidin/TNFa/DMSO
-1.5
-1
-0.5
0
0.5
1
1.5
2
Fold change

c.1


Estrogen (late)
17β-estradiol study 11
TNF-α/Il-1b/IL-6/PGE2
BMP-2 (late)
Myocardial infarction (7 d)/unstable angina (7 d)
Dilated cardiomyopathy (before VAD)
Sapphyrin PCI-2050
Sangivamycin
R547 study 6
R547 (24 h)
-1.5
-1
-0.5
0
0.5
1
1.5
Fold change

c.2

3.4.2.2. Transporter modulation from disease

The following disease were considered as modulators for the genes of interest: cardiovascular and metabolic disease (such as infarction and diabetes) and affection of the gastrointestinal organs (such as ulcerative colitis).

Overall the transporters of interest were not greatly affected from these conditions (fig. 3-5 to 3-8). Among the transporters, the one affected by the largest number of disease was OATP4C1, which was mainly up-regulated from a range of cardiovascular pathologies. The least affected transporter resulted to be OAT3, which only responded to diabetes, with a 1.34-fold decrease in T-cells. The overall pattern of regulation varies from one transporter to another and from one cell line to another when considering affections of the gastrointestinal organs. Transporters were mainly up-regulated, when looking at the effect of cardiovascular disease. Identifying which transporters are responsible for the inter-membrane transport of specific compounds would be a crucial step in improving post infarction treatment.

3.4.3. Discussion and conclusions

Modulation of transporters impacts on every biological process occurring within the cell. Understanding what compounds and how they affect expression of transporters could represent a vital tool to control selected cell functions. The final aim would be to enhance cellular health, by maximising health-promoting biological processes and minimising processes linking to cell damage and death.

In this review the current knowledge on the anatomical distribution of selected transporters (OAT1, OAT3, OATP1A2, OATP1B1, OATP1B3, OATP4C1, MRP2, MRP3, BCRP, MCT1, MCT7, SMCT1) was investigated for the human heart, liver, kidney, and intestine.

Moreover, the up to date information on the transcriptional modulation of these transporters was reviewed. The information reviewed in the present work was obtained using the high-performance search engine Genevestigator. Genevestigator contains information on the gene expression and modulation of a large number of genes and for multiple organisms, presented separately. All the data come from microarray experiments.

The given information is a start point for future studies on transporter modulation and gives an overall picture of the sensitivity of each transporter to a range of selected conditions (chemicals and drugs, disease, growth factors and cytokines, hormones).

Abundance of transporters within the organs of interest varies significantly. Some transporters, such as SMCT1, were ubiquitously expressed at low levels. Some others were localized in specific organs or organ parts, such as OATP1A2, which was only expressed in the heart and intestine.

Certain drugs displayed an opposite effect on uptake and efflux transporters. An example of that was given by treatment with zalypsis, an antibiotic with anti-neoplastic activity (Ocio *et al.*, 2009, Colado *et al.*, 2011). Zalypsis showed to up-regulate uptake transporters, such as OAT1, and down-regulate efflux transporters, such as MCT1.

It is worth of note the effect of PMA, observed when treating ASPC-1 cells. After treatment with PMA, the efflux transporters BCRP and MRP2 showed up-regulation. PMA triggered down-regulation of the uptake transporter OATP4C1, expressed in lymphocytic cells. PMA is a tumour-promoting agent (Yu *et al*., 2012), responsible for longevity of the neoplastic cells and for the vascularisation of cancerous tissues. In terms of cell physiology, the double effect of decreasing uptake and increasing efflux may explain the protective effect that PMA has on malignant cells and the resulting failure of anti-neoplastic agents when cells have been pre-exposed to PMA (Skinnider *et al.*, 1984).

Interestingly, both the efflux transporter MCT1 and MCT7, expressed in ASPC-1 cells, were down-regulated by PMA. This apparent contradiction in the effect of PMA could be explained with the different roles of the transporters in physiological cell functions and with different substrates transported.

Hormones, cytokines and growth factors are biological mediators known to play a major role from the early stages of human development. Therefore, being capable to identify the impact they have on transporters would represent a tool to control organ and tissue development and to fight a variety of diseases and dysfunctions. Examples of biological mediators are the TGF-α and TGF- β, which are involved in the regulation of many processes such as cell growth, immunity and cancer development (Kuranami *et al.*, 1989, Erbas and Lai, 2000). This review showed that transporters responded differently to biological mediators, according to their role in the cell physiology. For example, the uptake transporter OATP1B3, expressed in metastatic mammary gland cells, showed down-regulation following treatment with the SDF-1. The SDF-1 is a cytokine responsible for angiogenesis (Zhi *et al.*, 2011), it is a crucial factor in the neovascularisation of cancerous tissues and it is believed to be responsible for metastasis formation (Gellmini *et al.*, 2008). Major down-regulation of uptake transporters in response to SDF-1 could be associated with a decrease in the amount of chemotherapy drugs reaching the cancerous cells and thus with the resistance phenomena that lead to therapy ineffectiveness.

An example of up-regulation was observed on the uptake transporter OATP4C, after treatment of leukocytes with the G-CSF, a factor responsible for production of granulocytes and stem cells and for the proliferation and differentiation of neutrophils (Rolando *et al.*, 2000). In terms of cell physiology, such effect agrees with the proliferation of new cells,

which is strictly linked to the necessity for more compounds to be transported into the dividing cells.

When looking at the effect of disease, a consistent pattern of regulation was not observed. However, transporters were mainly up-regulated from cardiovascular disease. *In vivo* heart failure is linked to increased susceptibility to inflammatory diseases. This links to production of high levels of inflammatory molecules and subsequent cell damage (Fuchs and Drexler, 2004). It is possible that the physiological changes induced from heart failure are associated with an increased production of toxic compounds which is followed by increased efflux and transport of pro-inflammatory mediators out of the cell in the blood stream. This would finally contribute to promote inflammation.

The initial aim of creating the present comprehensive database was to identify one or more transporters standing out for their response to food compounds. This information would have been a starting point in the choice of a target transporter and in testing further food compounds. Unfortunately, despite the large amount of data existing about the modulation of transporters in response to chemicals, not many food compounds had been tested as gene modulators, indicating a lack of knowledge associated with this increasingly important area of study. The only food components used were curcumin and apple procyanidin, either used alone or after pre-incubation with the stressors H2O2 or TNF-α. In the first study, U937 cells were used to assess whether curcumin could revert the effect of hydrogen peroxide on selected genes. Cells were incubated with hydrogen peroxide for four hours and subsequently incubated for four hours with concentrations of curcumin up to 10 μM. Gene levels were investigated through microarrays before and after incubation with curcumin. Among the genes investigated were the 12 transporters of interest in this review. Interestingly only OATP1A2 and OATP1B1 expression was affected by hydrogen peroxide

and/or curcumin treatment. In the case of OATP1A2, hydrogen peroxide did not affect the expression levels, however treatment with curcumin up-regulated the mRNA levels by 1.12-fold. In the case of OATP1B1, hydrogen peroxide up-regulated the gene by 1.23-fold, while treatment with curcumin completely reverted this effect. In the second study, HUVEC cells were incubated with the TNF-α for four hours and subsequently incubated with 10 μg/ mL apple procyanidins for four more hours. Microarray analysis was performed to assess the response of selected genes to the stressor and subsequently to the polyphenol. Among the genes investigated were the 12 transporters of interest in this review. Only three of these transporters responded to treatment with apple procyanidins, BCRP, SMCT1 and MCT7. The TNF-α up-regulated BCRP by 1.23-fold and down-regulated SMCT1 by 1.18-fold, subsequently apple procyanidins completely reverted this effect. In the case of MCT7, the stressor did not affect the gene expression, while treatment with apple procyanidins up-regulated the mRNA by 1.56-fold. It was interesting to note that curcumin and apple procyanidins reverted the gene expression of selected uptake and efflux transporters, respectively. These two articles confirm the activity of polyphenols as antioxidants. It is possible that they have little effect on healthy cells, but major effect when oxidative stress is triggered. It is possible that modifications of the cell physiology, triggered by oxidative stress, link to activation of protective pathways involving polyphenols, as oxidative stress suppressors. A proposed mechanism for the activity of polyphenols is that they bind to specific factors, involved in protecting the cell against oxidative stress. This hypothesis would be in agreement with the *in vivo* study from Cao *et al.* (Cao *et al.*, 2007), assessing the effect that phenolic green tea extract have on glucose transporters (Glut1/*Slc2a1*, Glut2/*Slc2a2*, Glut3/*Slc2a3*, and Glut4/*Slc2a4*). In this study rats were fed for 6 weeks green tea extracts (1 or 2 g/kg diet) and their diet was supplemented with high levels of

fructose levels, a known inducer of oxidative stress (Blond *et al.*, 2011). The liver was then dissected and used to investigate the transporters of interest through RT-PCR. Results showed that 1 g of green tea/kg diet increased Glut1 and Glut4 expressed in the liver by 110 % and 160 % respectively, while 2 g of extracts increased Glut4 mRNA levels found in the liver by 90 % and Glut2 and Glut4 mRNA levels found in the muscle by 80 % and 40 %, respectively.

Uptake and efflux transporters have gained increasing attention in the last decades (Page *et al.*, 2001). Further analysis of transporter function(s) and mechanism(s) of action, in response to modulatory conditions, are necessary. This review is a starting point to better understand some of these objectives.

A comprehensive database, reporting anatomical distribution of the selected transporters and the conditions modulating the genes of interest can be found in Appendix I and II.

3.5. Conclusions

The present review aimed to collect information on the anatomical distribution and modulation of 12 transporters of interest. Some of the transporters resulted to be ubiquitously expressed in the human organs investigated (kidney, liver, heart and intestine), such as BCRP. Some others were only significantly expressed in one specific tissue, such as OAT1, which was only expressed in the kidney proximal tubules. Transporters responded differently to a range of conditions. Interestingly not many food compounds had been tested as modulators. The only two food compounds tested were curcumin and apple procyanidins. They were both used to revert the effect of stressors (hydrogen peroxide and the tumor necrosis factor α, respectively). It was observed that the two polyphenols were able to revert the effect of the stressors. This was at the basis of the next chapter, where stressors

(hydrogen peroxide, ethanol and *tert*-butyl-hydroperoxide) would be used as potential modulators of the organic anion transporter 3. The aim of the next chapter would be to identify stressors acting as modulators of OAT3 and subsequently to identify polyphenols able to revert the effect of the stressors.

Chapter 4

Modulation of the Organic Anion transporter 3 and assessment of kaempferol- 3-*O*-glucuronide uptake in liver model HepG2 cells

4.1. Abstract

The aim of the present work was to assess the effect of selecting stressing agents on the organic anion transporter 3 (OAT3) and to characterise the uptake mechanism(s) of kaempferol-3-*O*-glucoronide in the liver model HepG2 cells.

Expression of OAT3 in the cell model was initial assessed, through RT-PCR. The work showed for the first time that OAT3 is expressed in HepG2 cells.

Subsequently the effect of selected stressors (hydrogen peroxide, *tert*-butyl-hydroperoxide and ethanol) on the expression and function of OAT3 was assessed. Cells were treated with the stressing agents for 8-12 h. Subsequently, in order to assess expressional modulation of OAT3, RNA extraction was performed and RT-PCR carried out. Alternatively, to assess functional modulation, cells were incubated with a model substrate (5'-carboxyfluorescein), after the incubation with the stressing agents. Intracellular levels of 5'-carboxyfluorescein were measured through HPLC and compared to the levels in control, untreated, cells. None of the stressors showed to affect the expression and/or function of OAT3.

In order to characterise the uptake mechanism(s) of kaempferol-3-*O*-glucuronide in HepG2 cell, LC-MS was used to measure the metabolite uptake. Time-, concentration- and temperature-dependency of the uptake were assessed. Kaempferol-3-*O*-glucuronide showed to be mainly taken up *via* active transport (Km and Vmax were 143 µM and 2.2 pmol/min respectively).

4.2. Chapter summary

- The first aim of the present work was to determine whether the liver model HepG2 cells expressed OAT3. RT-PCR was used. The Ct value for OAT3 was 33, while the Ct value for the housekeeping gene GAPDH was 16, suggesting a low expression profile for OAT3.
- Subsequently it was investigated whether selected stressing agents modulated OAT3 expression and/or function in HepG2 cells.

  The stressing agents selected were hydrogen peroxide, *tert*-butyl-hydroperoxide and ethanol. The stressing agent concentrations to be used were chosen on the basis of preliminary cytotoxicity assessments, using two methods: the lactate dehydrogenase assay and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

  RT-PCR was performed to assess expressional modulation. Functional modulation was assessed through intracellular quantification of the model substrate 5'-carboxyfluorescein, through HPLC.

  None of the tested stressing agents were shown to modulate OAT3 expression or function.
- The final aim of the work was characterise the uptake of the metabolite kaempferol-3-*O*-glucuronide in HepG2 cells. Time-, concentration- and temperature-dependence of the uptake were assessed, by measuring the intracellular levels of the metabolite through LC-MS. It was shown that kaempferol-3-*O*-glucuronide was taken up in a time-, concentration- and temperature-dependent manner. These results suggested that kaempferol-3-*O*-glucuronide uptake most likely occurred *via* active transport. The Km and the Vmax were 143 μM and 2.2 pmol/min respectively.

4.3. Introduction

4.3.1. Role of human liver in detoxification

The liver is the main organ for detoxification in the body. A large and complex range of enzymes present in the liver transforms fat-soluble toxins into water-soluble ones. The latter can be eliminated in the urine or bile, according to the specific features of the end-product. As a primary site of detoxification, the human liver plays a major role in maintaining cell integrity (Ramaccioni *et al.*, 2000, Schmidt *et al.*, 2010).

It is important to understand what are the modifications triggered by oxidative stress on the liver cell physiology and how dietary compounds can be determinant in maintaining the balance and ensuring correct functioning of the hepatocytes (fig. 4-1).

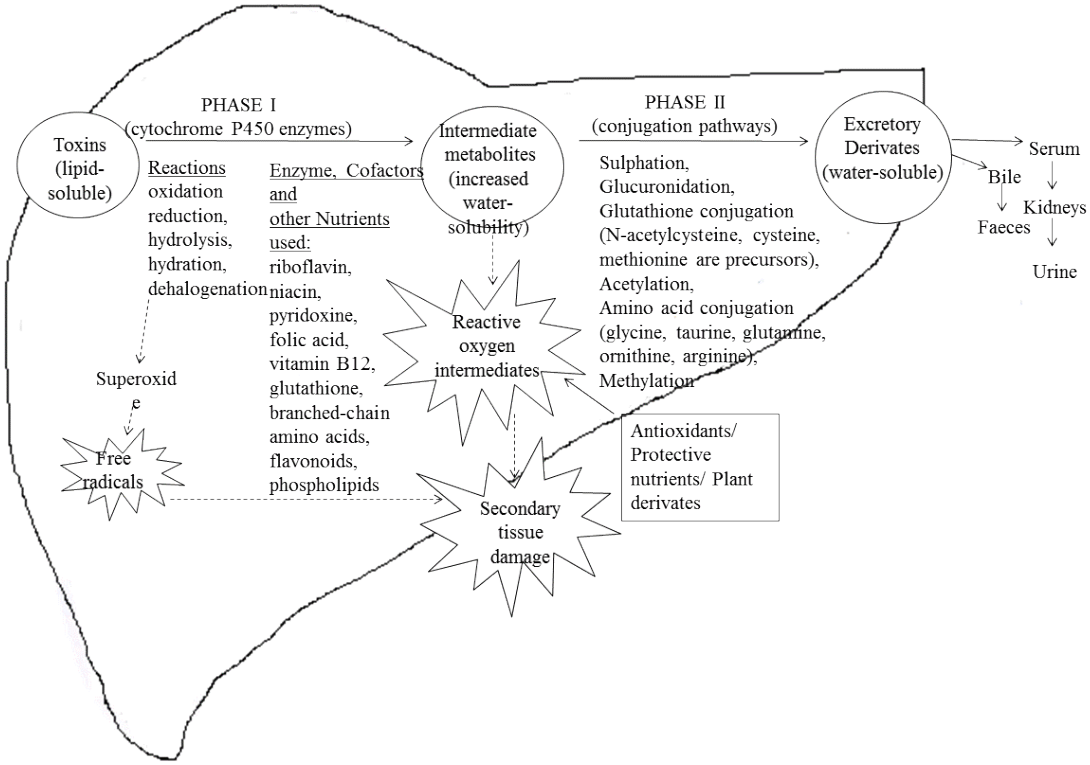


Figure 4-1. Schematic representation of the human liver, showing its functions and role in nutrient and non-nutrient processing and detoxification.

Oxidative agents, such as hydrogen peroxide (H_2O_2) and *tert*-butyl-hydroperoxide (*t*-BOOH), have been proved to cause cell damage and, in case of prolonged exposition, degenerative disease and death (Gutierrez-Ruiz *et al.*, 1999, Van de Bittner *et al.*, 2010, Fedotcheva and Mokhova, 2013). It is been hypothesized that oxidative stress impacts on cell health in various ways including by affecting transporter expression and/or activity (Ikemura *et al.*, 2009).

A wide range of uptake transporters is expressed in the human hepatocytes. Among them are several members of the OATPs and one member of the OATs, OAT2. OAT3 has been

shown to be highly expressed in the kidney but does not appear to be expressed in the liver (Hilgendorf et al., 2007a).

4.3.1.1. OAT3 gene structure and gene regulation

To better understand the biological processes leading to transporter modulation, an analysis of gene structure is crucial. The gene structure of OAT3 is not yet fully characterised (fig. 4-2).

Previous published work on OATs exist, investigating the gene sequences and their regulation. Regulation of the OAT3 gene, *SLC22A8*, has been previously investigated (Ogasawara *et al.*, 2006). Cells from opossum were used to clone the *SLC22A8* promoter and to perform functional promoter assays. The study showed that both basal and inducible activity of the *SLC22A8* promoter depended on the activity of the cAMP response element (CRE). Two transcription factors were also involved, CREB-1 (cAMP response element binding 1), and ATF-1 (activating transcription factor 1). They were shown to act by dimerising and binding to CRE.

PKCs (protein kinase C) are isozymes involved in signal pathways regulating many processes, such as cell growth (Hirai and Chida, 2003). Different second messengers are required for their activation. On the basis of the second messengers required, the PKCs can be divided in three types: classical, novel and atypical.

A study on gene regulation of organic anion transporters showed that OAT1 and OAT3 were up-regulated through a pathway involving PKCζ, an atypical PKC (Barros *et al.*, 2009). The experiment was carried out using two sets of renal cortical tissue slices from rats over-expressing OAT3 and OAT1 respectively. When cells were treated with insulin, a PKCζ inducer, OAT1- and OAT3-mediated uptake increased. In cells treated with a PKCζ

inhibitor, transport of OAT1 and OAT3 substrates was inhibited. This confirmed PKCζ-dependent activation of OAT1 and OAT3.

The authors also assessed the role of ATF-1 in gene expression. They showed that ATF-1 alone did not activate gene expression, despite dimerising with CREB-1. In contrast, gene expression was activated when ATF-1 combined with PKA. It was also shown that phosphorylation of CREB-1 and ATF-1 by PKA resulted in increased promoter activity and gene expression.

A study published in 2006 (Kikuchi *et al.*, 2006) showed the crucial role of the human nuclear factor 1 (HNF1) in regulating the expression of OAT3. HNF1 is a homeodomain-containing factor, consisting of two isoforms, HNFα and HNFβ. HNF1 is expressed in a wide range of organs, including liver and kidney (Saji *et al.*, 2008). In the study, promoters were used to identify the functional region of the *SLC22A8* promoter. It was shown that the region between -308 and +6 is essential for promoter activity. Homology of this region between different mammalian (human, rat and mouse) was also investigated, showing that almost 70 % homology exists. The authors assessed that the structure of the two isoforms of HNF1 enabled, with the same probability, both homodimer and heterodimer formation. Both complexes are likely to form *in vivo*. However, the transactivation potency of HNFα was shown to be higher than that of HNFβ. In this study the importance of promoter methylation for gene expression was also investigated. The authors analyzed different tissues, some expressing OAT3 and some not expressing it, and observed that the gene was present in both tissues. However they observed that in the tissues not expressing the corresponding protein, the gene promoter was hypermethylated. In contrast, in the tissues expressing the protein, the promoter was hypomethylated. Authors concluded that methylation is a key mechanism to control tissue-specific gene expression.

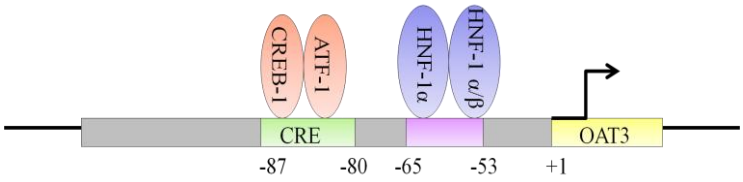


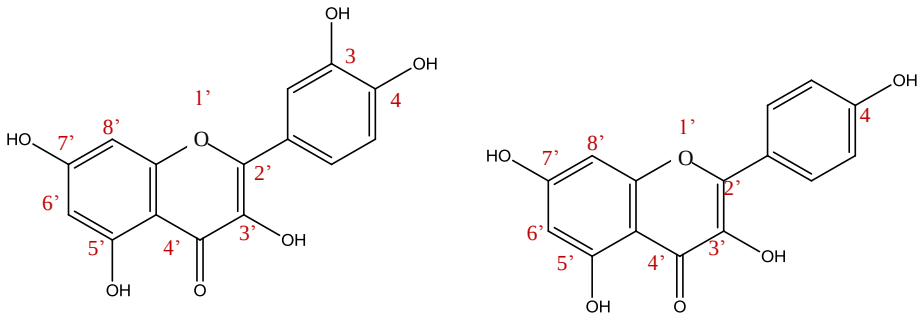
Figure 4-2. Characterized promoter regions on the OAT3 (*SLC22A8*) gene promoter.

4.3.2. Kaempferol-3-*O*-glucuronide uptake in HepG2 cells

4.3.2.1. Kaempferol: metabolism and impact on human health

Kaempferol (fig. 4-3) is a flavonoid abundant in a variety of foods. Examples of kaempferol sources are tea, broccoli, grapefruit, cabbage and strawberries. The mean intake for kaempferol has been determined to be around 4.7 mg/day (Radtke *et al.*, 2002).

Reports showed that among flavonols, kaempferol is the second biggest contributor to the human diet, after quercetin (Sampson *et al.*, 2002). Preclinical studies have suggested that kaempferol and its metabolites are associated with a range of pharmacological activities, such as anti-cancer, cardio-protective and anti-inflammatory activities (Jeong *et al.*, 2009, Sun *et al.*, 2013). An *in vivo* study showed a significant decrease in pancreatic cancer risk in smokers consuming kaempferol, quercetin and myricetin on a daily basis for eight years (Nothlings *et al.*, 2007). Kaempferol and quercetin have high structural similarity, the only difference is that quercetin has two hydroxyl group substitutions (3 and 4) on the 2' benzyl group, while kaempferol has only one hydroxyl group (4) on the 2' benzyl group (Fig. 4-3).



Quercetin **Kaempferol**

Figure 4-3. Chemical structure of quercetin and kaempferol.

Whilst numerous studies have focused on understanding the mechanisms of quercetin uptake and efflux (Petri *et al.*, 2003, Scalia *et al.*, 2013, Glaeser *et al.*, 2014), little is known about kaempferol and kaempferol metabolite transport in and out of cells or about their bioavailability (Liu *et al.*, 2006).

Kaempferol in nature is often found in the form of glycosides. The relative abundance of these glycosides varies between members of the pant kingdom (Iwashina *et al.*, 2010).

After kaempferol ingestion, metabolism of this compound is extensively carried out in the liver. Gluco- and sulfo-conjugates are predominantly produced. It has been shown that the most abundant kaempferol metabolite to be retrieved from human urine is kaempferol-3-*O*-glucorinide (fig. 4-4) (DuPont *et al.*, 2004).

It has been shown that 1.9-2.5 % of the total ingested kaempferol is excreted in the urine (Calderon-Montano *et al.*, 2011). A study carried out on eight volunteers showed that the maximum plasma concentration for kaempferol was reached after 5.8 hours from ingestion (DuPont *et al.*, 2004). In this study volunteers were provided with a portion of endive, containing 8.65 mg kaempferol present as a mixture of 3-glucuronide, 3-glucoside and 3-(6-malonyl)-glucoside (79 %, 14 % and 7 % respectively). Volunteers were healthy non-

smokers. In all the volunteers it was observed that the maximum blood concentration of kaempferol was 100 nM.

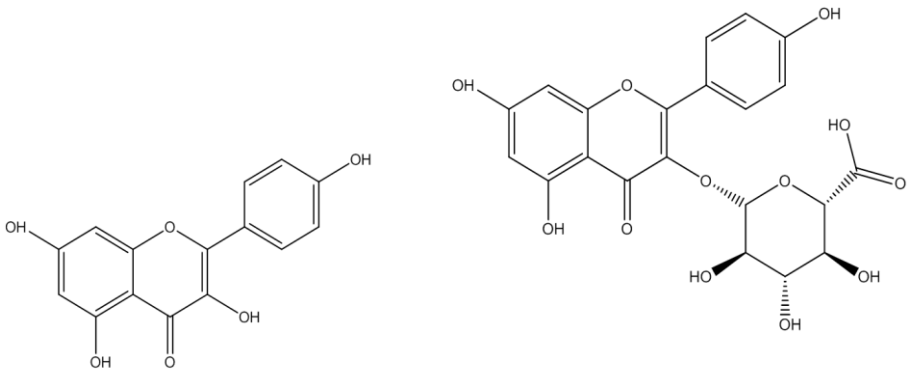


Figure 4-4. Chemical structure of kaempferol and kaempferol-3-*O*-glucuronide.

Not much is known on how kaempferol metabolites enter the cell and whether they are transported actively through carrier proteins or move mainly through mechanisms of passive diffusion. It is important to assess the mechanisms behind the cellular uptake of these components and what are the biological consequences of decreased/increased levels of these putative bioactive compounds.

4.4. Results

4.4.1. Assessment of the expressional modulation of OAT3

4.4.1.1. Assessment of OAT3 gene expression and modulation in liver HepG2 cells

In the present study, RT-PCR was carried out to assess whether the OAT3 gene, *SLC22A8*, was expressed in liver model HepG2 cells. OAT3 gene (*SLC22A8*) was found in HepG2 cells. A low expression profile for the gene was predicted, using the Ct value. The Ct value for *SLC22A8* was 33 (see chapter 2, fig. 2-3), when using 100 ng/µL cDNA. Using the same concentration of cDNA, the Ct value for the housekeeping gene GAPDH was 16.

Cells were treated with different stressing agents for 12 h and subsequently incubated with 5'-CF, a model substrate for OAT3. RT-PCR was performed to assess whether the stressors could act as expressional modulators of *SLC22A8*. It was shown that none of the stressors induced changes in the expression of *SLC22A8* when comparing gene expression between stressor-treated and untreated cells (fig. 4-5).

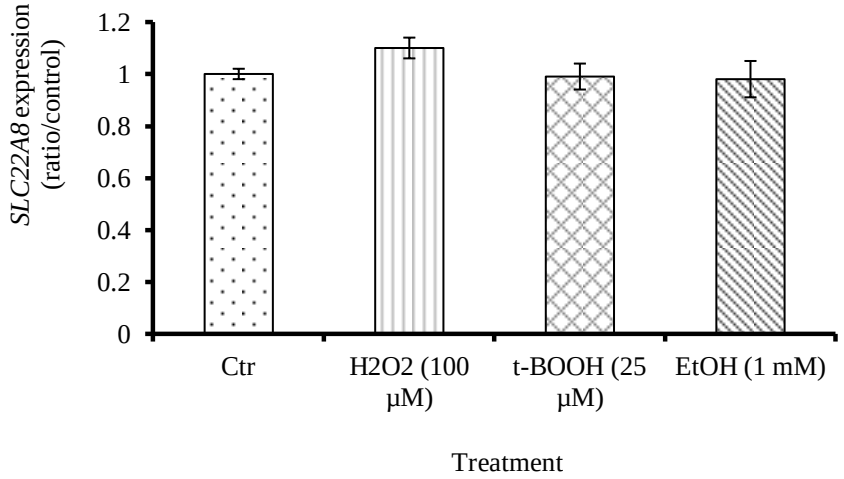
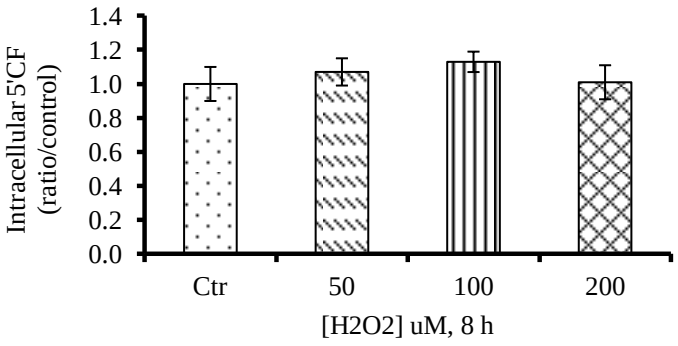
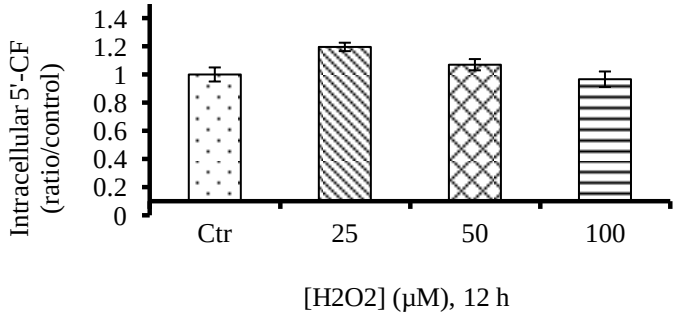


Figure 4-5. Gene expression modulation of OAT3 (*SLC22A8*) using different stressing agents. HepG2 cells were seeded in 6 well plates and grown until confluent. Cells were incubated for 12 h at 37 °C with H_2O_2 (100 µM), t-BOOH (25 µM) and EtOH (1 mM). After incubation, RNA was extracted and RT-PCR performed. Results are expressed as the ratio of the expression levels of *SLC22A8* from cells treated with each stressor over expression levels from control un-treated cells. Results represent average of triplicates ± St. Dev.

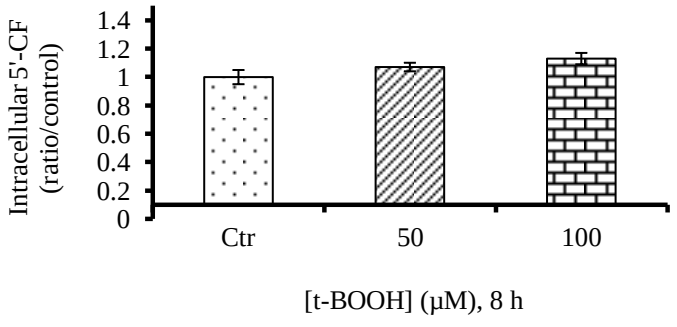
The effect of each stressor on OAT3 function was evaluated. Initially the cells were incubated with each stressor for 8 or 12 h and subsequently with 5'-CF for 10 min. Intracellular 5'-CF content was evaluated through HPLC and compared to un-treated cells. No statistically relevant changes were detected (fig. 4-6).



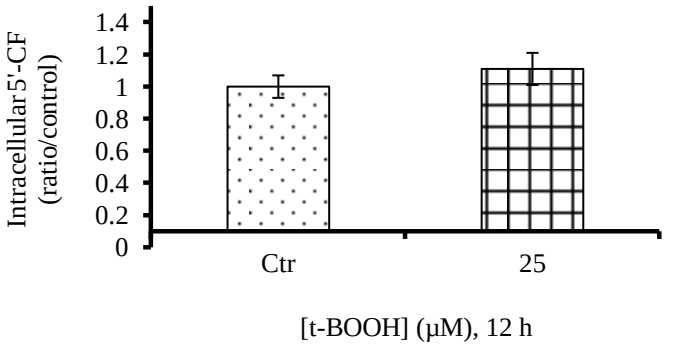
a.



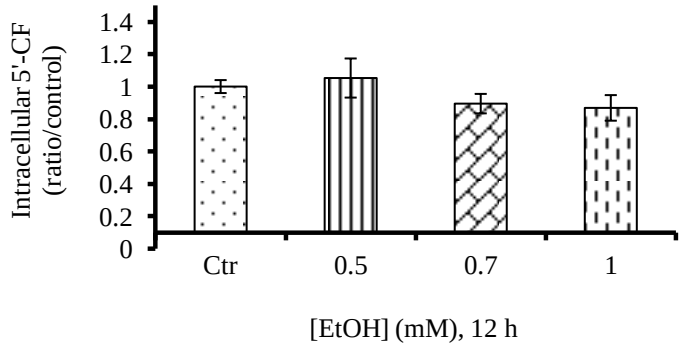
b.



c.



d.



e.

Figure 4-6. Functional modulation of OAT3 from stressing agents. HepG2 cells were seeded in 6-well plates and grown until confluent. Cells were incubated for 8 or 12 h at 37 °C with H_2O_2, t-BOOH and EtOH respectively. Subsequently cells were incubated for 10 min at 37 °C with 100 µM 5'-CF. Cells were lysed and the intracellular content was collected. 5'-CF was quantified through HPLC. Results are expressed as ratio of the intracellular 5'-CF content from stressor-treated cells over 5'-CF content from un-treated control cells. Results represent average of triplicates ± St. Dev.

4.4.2 Kaempferol-3-O-glucuronide uptake

Intracellular k-3-*O*-g was evaluated, using a k-3-*O*-g standard curve (fig 4-7). Time-, concentration- and temperature-dependence of uptake were evaluated by measuring the intracellular k-3-*O*-g through LC-MS. Uptake was shown to be time- (fig. 4-7), concentration- (fig. 4-8) and temperature- (fig. 4-9) dependent.

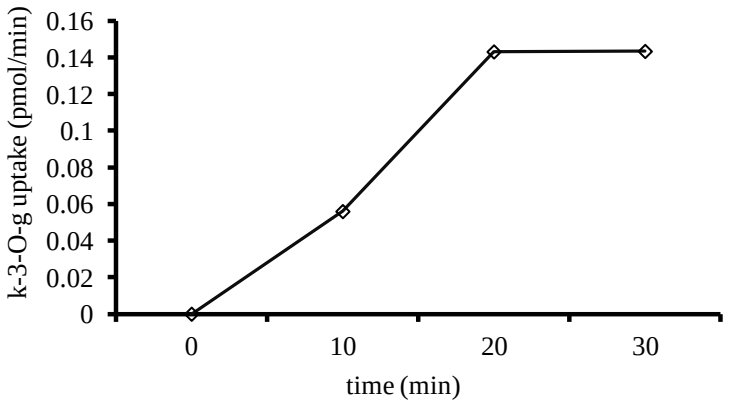


Figure 4-7. Time-dependent uptake of kaempferol-3-*O*-glucuronide in HepG2 cells. HepG2 cells were seeded in 6-well plates and grown until confluent. Cells were incubated with 55 μM k-3-*O*-g at 37 °C for 10-30 min. Cells were lysed and each well content collected separately. Each well content was analysed through LC-MS, to determine the metabolite uptake in each well. The intracellular k-3-*O*-g content for each well was calculated in pmol, using a standard curve prepared with k-3-*O*-g authentic standards. Subsequently the calculated amount of intracellular k-3-*O*-g was divided for the incubation time, obtaining the uptake rate per minute (pmol/min). Representative results are displayed.

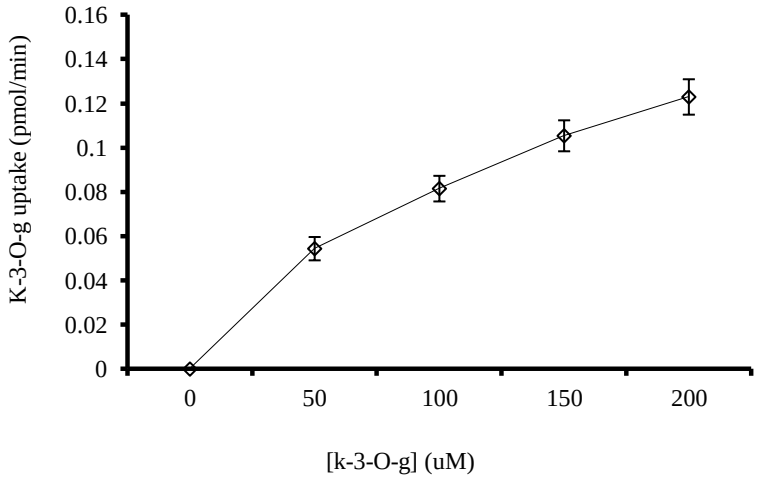


Figure 4-8. Concentration-dependent uptake of kaempferol-3-O-glucuronide in HepG2 cells. HepG2 cells were seeded in 6-well plates and grown until confluent. Cells were incubated with 50-200 µM k-3-*O*-g at 37 °C for 10 min. K-3-*O*-g uptake was quantified through LC-MS. Uptake is expressed as pmol/min. Results represent average of triplicates ± St. Dev.

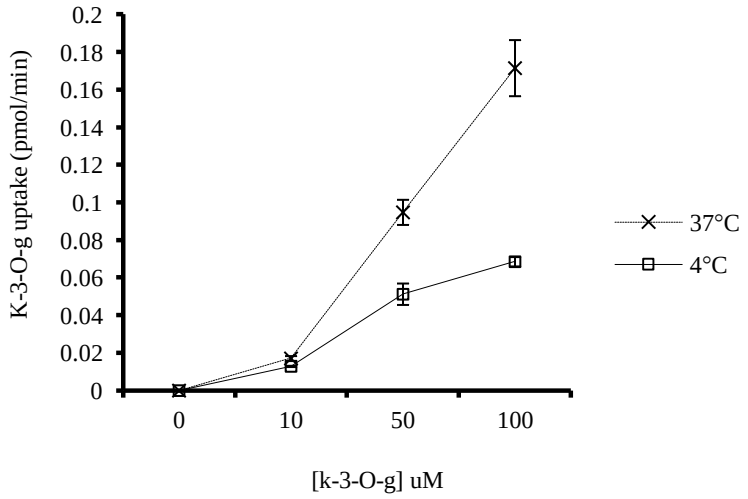


Figure 4-9. Temperature-dependent uptake of kaempferol-3-*O*-glucuronide in HepG2cells. HepG2 cells were seeded in 6-well plates and grown until confluent. Cells were incubated with 10-100 µM k-3-*O*-g at 37 °C for 10 min. K-3-*O*-g uptake was quantified through LC-MS. Results represent average of triplicates ± St. Dev.

Results from LC-MS analysis were used in the Lineweaver-Burk plot to calculate Km and Vmax for k-3-*O*-g uptake. Km and Vmax were 143 µM and 0.22 pmol/min respectively (fig. 4-9).

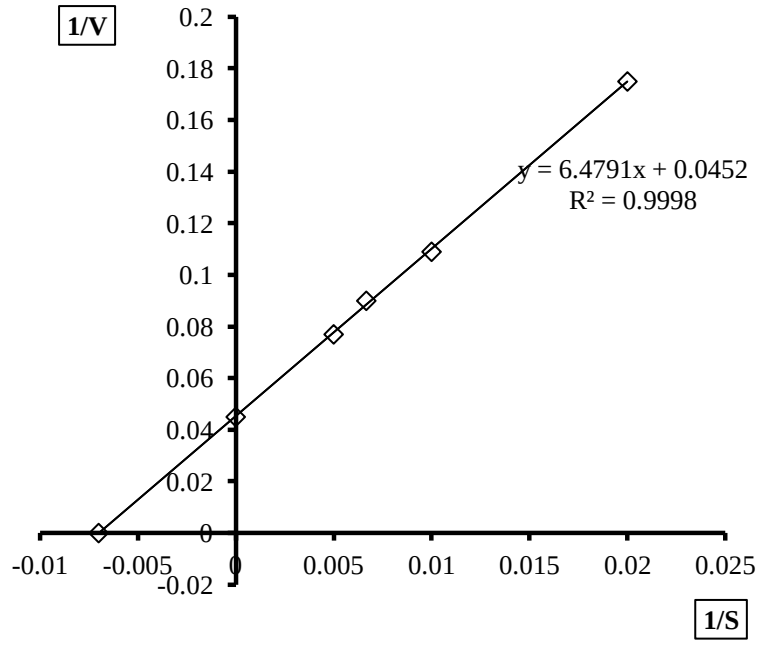


Figure 4-10. Calculation of Km and Vmax for k-3-O-g uptake in HepG2 cells. The Lineweaver-Burk plot was used to calculate Km and Vmax, where S = initial substrate concentration and V = uptake/min (pmol/min). From the equation: Km = -1/x intercept = -1/(-0.4548/64.954) = 143 µM and Vmax = 1/y intercept = 1/0.4548 = 0.22 pmol/min.

4.5. Discussion and future prospectives

4.5.1. Modulation of OAT3 using stressing agents

The present study focused on better understanding the uptake mechanisms and the uptake modulation in liver model HepG2 cells. Two different aspects of uptake were investigated: the expressional and functional modulation of the uptake transporter OAT3, and the uptake mechanism(s) for the metabolite kaempferol-3-*O*-glucuronide.

The study showed for the first time presence of the OAT3 gene, *SLC22A8*, in HepG2 cells. Low levels of expressions were predicted on the basis of the Ct value. Stressing agents (H_2O_2, t-BOOH and EtOH) were tested as potential modulators of OAT3. The stressing agents were tested at ranges of concentrations selected using the LDH and MTT cytotoxicity tests. Two different experiments were carried out to assess modulation of OAT3 gene (*SLC22A8*) and of the OAT3 protein respectively. The gene modulation was assessed using RT-PCR to assess whether the mRNA levels for *SLC22A8* increased/decreases in response to long incubation (8-12 h) with each stressor. None of the tested stressors showed to affect *SLC22A8* amount. Modulation at protein level was assessed by incubating the cells with each stressor and subsequently quantifying the uptake of a model substrate for OAT3, 5'-CF, through HPLC. None of the tested stressors affected OAT3 function, decreasing or increasing the amount of 5'-CF taken up in the cell model.

Multiple studies have been carried out, assessing expression and distribution of OAT3 (*SLC22A8*) across human tissues (Sun *et al.*, 2001, Anzai *et al.*, 2006). All the published data report that the main site of expression of OAT3 is the proximal tubule of the kidney. A study (Hilgendorf et al., 2007a) assessed the expression of 36 transporters in human cell lines and tissues. Expression was assessed in cell lines and human tissues, representative of intestine, liver and kidney. The study reported a great difference between HepG2 cells and

liver tissues, highlighting how these cells may not be the best candidates to select when modeling the human liver. The paper also reported a lack of expression of *SLC22A8* in both liver tissues and HepG2 cells. This is in contrast with the present study that shows low expression of OAT3 in HepG2 cells.

Data differences between different laboratories are not uncommon. Examples exist in the literature, showing both qualitative and quantitative discrepancies in the expression of transporters (Jennings *et al.*, 2009, Phillips and Castegnaro, 1999). Differences can depend on multiple factors, such as the methodology used for mRNA quantification and the experimental design (Sun *et al.*, 2002, Landowski *et al.*, 2003). When carrying out experiments using cell lines, one of the possible explanations for the inter-laboratory discrepancy may be the use of different cell passages for the experiments. Studies have shown that different cell stages are linked to different gene expression patterns and different cell behaviors, associated with different cellular developmental stages (Kerber and Cawthon, 2007, Middleton *et al.*, 2013).

An important consideration to be made is that in the present study an absolute quantification of the *SLC22A8* mRNA levels was not made. The Ct value does not represent an absolute measure of the target gene, but is rather used to indicate presence or absence of the latter (Pornprasert *et al.*, 2010). The Ct calculation is also affected from a wide range of conditions, such as the Master Mix used (Sayler *et al.*, 2011). It would have been interesting to compare the Ct value for *SLC22A8* in HepG2 cells with other cell lines. This would have been useful to have an overall picture of the abundance of *SLC22A8* in different cell models.

Another limitation of the present study is the sole use of PCR to look at the expression of the gene of interest. The polymerase chain reaction only gives a relative measure of the target gene of interest in the sample. This technique does not give any information on the abundance of the corresponding protein (Shen *et al.*, 2007). It would have been useful to

quantify the corresponding OAT3 protein in HepG2 cells, for example through Western blot analysis. It would also be interesting to compare gene and protein expression of this transporter across multiple cell lines.

When modulation of OAT3 was investigated, none of the tested stressing agents (H_2O_2, t-BOOH and EtOH) was shown to act as a modulator. A previous study reported that H_2O_2 at concentrations between 0.01 and 1 mM acted as a powerful down-regulator of hOAT3. (Takeda *et al.*, 2000). Proximal tubule cells from mice (S_2) were used in this study. Cells had been transfected with hOAT3. The study reported a dramatic decrease in the uptake of estrone sulfate, a substrate of OAT3, after exposure to hydrogen peroxide. In contrast, the present work does not show similar results. The choice of a different cell system may have impacted on the outcome of the study.

Another study reported that t-BOOH acted as a potent modulator of OAT1 uptake in renal cortical slides (Roma *et al.*, 2008). The work investigated the effect of t-BOOH on p-aminohippurate (PAH) transport. It was shown that incubation with the oxidative agent permanently reduced uptake of the PAH. In chapter 1, it was discussed how OAT1 and OAT3 are closely related in terms of gene structure, mechanism of action and function regulation. However, it is possible that the different response of OAT1 and OAT3 to modulators could depend on the differences existing in the two gene promoters. For this reason, in the future it would be very interesting to fully characterize the two gene promoters. A comparison of the functional domains involved in gene up-/down-regulation would be interesting.

Overall these results are inconclusive and would require further investigations to clarify their biological meaning and implications. However, from what has been shown so far it is possible to hypothesize that OAT3 is not involved in detoxification and this is the reason why it is not affected from stressors. It would be interesting to carry out more experiments

to identify the main role(s) of OAT3 in human liver and health and to identify possible modulators of the transporter.

4.5.2. Characterization of kaempferol-3-O-glucuronide uptake

After observing that none of the selected stressors affected OAT3 gene levels and/or protein function, a change of approach was decided. The work focus was addressed on characterizing the uptake mechanisms(s) of a selected metabolite (kaempferol-3-*O*-glucuronide) in the liver HepG2 cell model.

The final aim was to assess transporter modulation in liver HepG2 cells. After having failed to identify modulators for the OAT3 gene (*SLC22A8*), a different aspect of transporter modulation was selected. The aim was to assess whether uptake of k-3-*O*-g mainly occurred *via* carrier-mediated transport or passive diffusion mechanisms. If k-3-*O*-g uptake resulted to be mainly relying on active carriers, the aim of this study would be to identify which carriers were involved. This would have been done using a range of strategies, such as selective gene knock-down and gene over-expression.

At the same time, addressing different aspect of gene modulation in HepG2 cells would give a better understating of the overall cell model validity for modulation experiments.

The present work showed for the first time that uptake of k-3-*O*-g most likely occurs through mechanisms of active transport (carrier-mediated transport relying on energy) in the liver model cells HepG2. However, it was shown that the uptake rate was very low. This made the cell model unsuitable for the assessment of k-3-*O*-g uptake modulation. The reason for that is that with the uptake rate being so close to detection limit, if uptake inhibition would be carried out, the intracellular amount of k-3-*O*-g would decrease to levels were it would no longer be detectable.

When kaempferol is ingested, it undergoes conjugation from phase II enzymes. This process results in a decreased ability of the compound to move across the biological membranes *via* free diffusion. Therefore it has been hypothesized that transport mechanisms relying on energy may be responsible for the uptake of kaempferol metabolites (Barrington *et al.*, 2009). Kaempferol is one of the highest contributors to flavonol intake in the human diet. Following consumption of kaempferol, glucuronides are among the main metabolites (O'Leary *et al.*, 2003, Barrington *et al.*, 2009). The present work showed that k-3-*O*-g uptake in HepG2 cells, at physiological concentrations, is temperature- and time- dependent. Moreover results showed that transport was concentration-dependent and saturable, with an estimated Km of 143 μM and Vmax of 0.22. pmol/min. The high structural similarity between quercetin and kaempferol has been described in the introduction session of this chapter. Studies have described that quercetin glucuronides undergo uptake and turnover in liver HepG2 cells (O'Leary *et al.*, 2003). Furthermore studies have shown that k-3-*O*-g is transported and further metabolized in hepatocytes by specific UDP-glucuronosyltransferase isoforms (Oliveira *et al.*, 2002). However no literature had previously investigated the mechanism(s) of uptake of k-3-*O*-g in liver cells, which was investigated for the first time in the present work.

In the present work it was shown that k-3-*O*-g uptake is most likely occurring via active transport (relying on energy use). Unfortunately inhibition experiments, aimed to identify which transporters are responsible for the uptake, were not possible due to the very low uptake rate.

A possible approach to characterize the carrier(s) involved, overcoming the problem of the low uptake rate, could be to carry out experiments linking to increased k-3-*O*-g uptake (Hennige *et al.*, 2003). An example would be to identify compounds acting as up-regulators of specific transporters expressed in HepG2 cells and to use them to treat the cells. The

repercussions on the intracellular levels of k-3-*O*-g would be assessed in response to cell treatment with each of the up-regulators. Once having identified possible transporter or transporter families responsible for uptake of the metabolite, overexpression experiments of the candidate transporters could be used to confirm that a selected transporter was responsible for the uptake.

Overall these results are important in highlighting a mechanism for kaempferol-3-*O*-glucuronide entry into liver cells, following kaempferol metabolism. More studies should be carried out to identify the protein(s) responsible for uptake of the metabolite.

The findings reported in the present wok, its limitation and the plans for future work, based on these findings, are further discussed in chapter 6.

4.6. Conclusions

The present work had three main findings: for the first time expression of the transporter OAT3 was shown in liver HepG2 cells, OAT3 did not show to be modulated from any of the tested stressors (hydrogen peroxide, *tert*-butyl-hydroperoxide and ethanol). Finally it was shown that the metabolite kaempferol-3-*O*-glucuronide was taken up in a concentration-, time- and temperature-dependant manner in the liver model HepG2 cells. However, modulating kaempferol-3-*O*-glucuronide uptake was not possible due to the low Vmax of uptake in the system (0.22 pmol/min).

Overall the cell line resulted to be a poor model for transporter modulation assessment. Therefore in the next chapter a different cell model was used (the intestine model Caco-2 cells).

Chapter 5

Effect of green tea and coffee on the serotonin reuptake transporter using the human intestine Caco-2 cell model

5.1. Abstract

Imbalance of serotonin levels in the gut is associated with a range of chronic pathologies. In this work green tea and coffee were investigated as modulators of serotonin uptake, using the human intestinal model Caco-2cells.

Whole green tea extracts displayed to inhibit uptake in a concentration dependent manner. Individual components (epigallocatechin gallate, epigallocatechin, epicatechin gallate, epicatechin) also displayed dose-dependent inhibition when tested at supplement concentrations (equivalent to 7 cups of green tea). Among the tested green tea components, epigallocatechin showed the most potent inhibition.

Coffee tested at physiological concentrations showed to be a potent dose-dependent inhibitor of serotonin uptake.

It was shown that among the coffee components the most potent inhibitor of serotonin uptake was 5-*O*-feruloylquinic acid. 5-*O*-feruoylquinic acid acted as a competitive inhibitor (K_i = 340 µM) accounting for over 50 % of the inhibition triggered from coffee.

Ferulic acid also inhibited uptake in a competitive manner (K_i = 270 µM), whereas caffeine was inactive.

The data predict that strong coffee could be almost as potent as the drug imipramine, used to treat patients with gut dysfunctions. The data also provide evidence for a novel caffeine-independent effect of coffee on gut serotonin-mediated signalling, involving chlorogenic acid constituents as well.

5.2. Chapter summary

- The aim of the present work was to investigate whether coffee and green tea could modulate serotonin uptake through the serotonin reuptake transporter (SERT) in intestinal model Caco-2 cells. The uptake of tritium-labeled serotonin was evaluated with and without the compounds of interest, using a scintillation counter.
- Coffee and green tea inhibited serotonin uptake in a concentration-dependent manner, with IC_{50} of 8.45 mg/mL and 10.9 mg/mL respectively.
- Individual green tea compounds were tested at physiological concentrations $\leq$ 1 mM. Epigallocatechin gallate (EGCG) did not affect serotonin uptake. Concentration-dependent inhibition was associated with epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC).
- Supplement concentrations (equivalent to 7 cups of green tea) of EGCG, EGC, ECG and EC inhibited uptake by 30, 54, 43 and 23 % respectively.
- Individual coffee components were tested at 1 mM. Caffeic acid (CA), ferulic acid FA), 3,4-dimethoxycinnamic acid (3,4-DMCIN), 5-*O*-feruoylquinic acid (5-CQA) and 3-*O*-caffeoylquinic acid (3-CQA) inhibited uptake by 17.4, 25, 26, 33 and 9 % respectively. 5-FQA and FA inhibited uptake in a competitive manner, with K_i = 340 and 270 µM respectively
- Caffeine, 3,4-dicaffeoylquinic acid (3,4-diCQA), 3,5-dicaffeoylquinic acid (3,5-diCQA) and 5-*O*-caffeoylquinic acid (5-CQA) had no effect on serotonin uptake
- Individual compounds from coffee and green tea could be used to create drugs modulating serotonin uptake in the gut. These drugs could be used to fight pathologies caused by serotonin imbalance.

5.3. Introduction

5.3.1. Synthesis and importance of serotonin for human health

The neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) is synthesized from the amino acid tryptophan (Fig. 5-1). About 90 % of the total serotonin is synthesised in the enterochromaffin cells of the gut (Best *et al.*, 2010). In the gut serotonin is involved in a range of processes such as nutrient absorption (Ellis *et al.*, 2013, Coates *et al.*, 2004), nociception (Maneepak *et al.*, 2009) and appetite modulation (Lima *et al.*, 2004, Gill *et al.*, 2008). Altered balance of serotonin in the gut is associated with a range of chronic gastrointestinal pathologies, such as irritable bowel syndrome and inflammatory bowel disease (Wheatcroft *et al.*, 2005, Wang *et al.*, 2008). In addition, imbalance of serotonin in the gastrointestinal tract has been linked to colorectal cancer (Chubak *et al.*, 2009, Ataee *et al.*, 2010) and celiac disease (Coleman *et al.*, 2006).

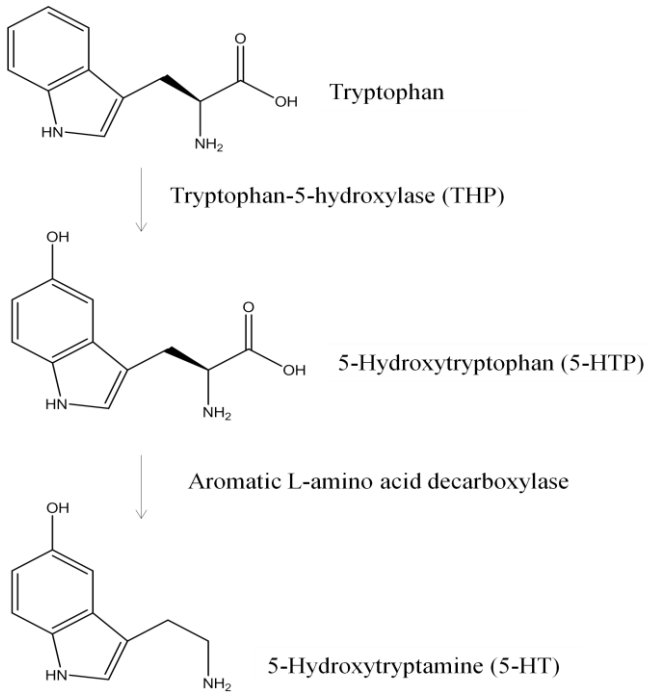


Figure 5-1. Steps of serotonin synthesis. Serotonin is synthesized from the amino acid L-tryptophan in two steps. Initial hydroxylation is catalysed by tryptophan-5-hydroxylase, producing 5-hydroxytryptophan. Subsequently 5-hydroxytryptophan is decarboxylated by the aromatic-L-amino acid decarboxylase, producing serotonin.

The role of serotonin has been best investigated in the central nervous system (Williams *et al.*, 2001), while less is known about the function(s) of serotonin in the human gut. It has been shown that the appetite suppressor fenfluramine partly acted by stimulating release of serotonin in the gut and that subsequently serotonin acted at brain level, reducing appetite (McCann *et al.*, 1995). Studies have also shown that alteration of tryptophan and serotonin balance at brain level were determinants in the development of food disorders such an *anorexia nervosa* (Curzon, 1990).

Serotonin action starts with release in the extracellular space. From here the neurotransmitter reaches target cells and activates the receptors exposed on the cell surface, initiating a cascade response. When serotonin dissociates from its receptors, action is terminated and serotonin is re-uptaken in the cell cytoplasm (Isaac *et al.*, 2013). Here the neurotransmitter is metabolised and recycled (fig. 5-2).

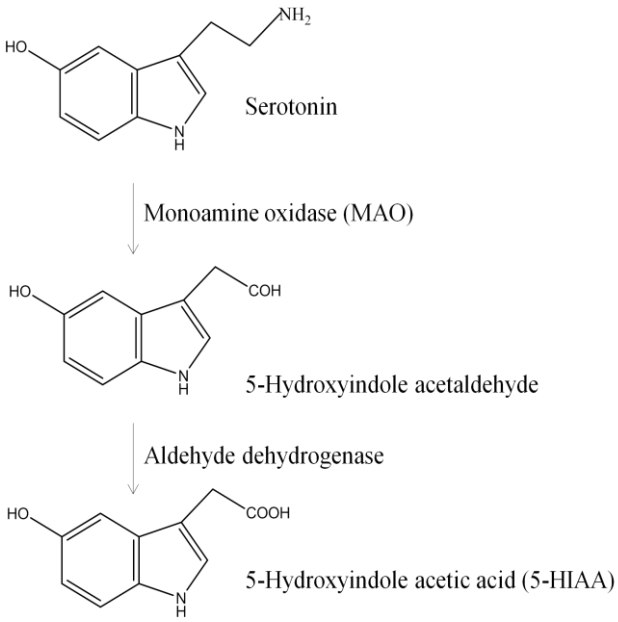


Figure 5-2. Metabolism of serotonin. After serotonin re-uptake in the releasing cell, the neurotransmitter is metabolised by the enzymes monoamine oxidase and aldehyde dehydrogenase into 5-hydroxyindoleacetic acid.

The sodium-dependent serotonin reuptake transporter (SERT) (fig. 5-3) contributes to serotonin re-uptake (Field *et al.*, 2010, Giannaccini *et al.*, 2011).

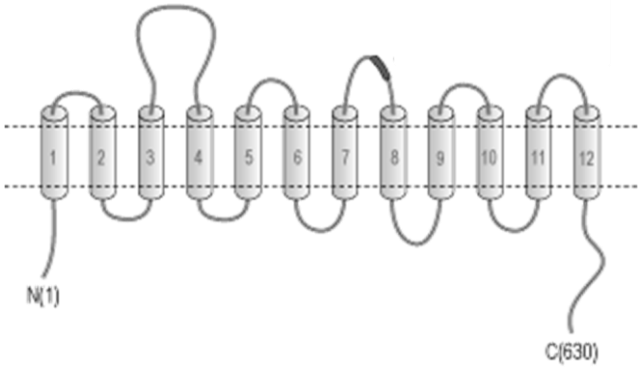


Figure 5-3. Structure of the serotonin reuptake transporter. SERT is a 12 transmembrane domain protein encoded by 630 amino acids. Domain 3 is crucial for serotonin translocation (Horschitz *et al.*, 2001).

Serotonin is released from the pre-synaptic cell in the synaptic space, where it reaches the receptors expressed on the surface of the post-synaptic cell. Serotonin binding to receptors initiate a response cascade in the cell. As soon as the response cascade is initiated, serotonin dissociates from the receptors and is taken back up into the releasing cell through SERT (Koldso *et al.*, 2011). Therefore SERT plays a determinant role in the intracellular/extracellular balance of serotonin. In pathologies caused by low serotonin release, such as certain forms of depression and irritable bowel syndrome, selective serotonin reuptake inhibitors are used to decrease the amount of serotonin taken back into the releasing cell (fig. 5-4). Selective inhibition of SERT results in increased levels of extracellular serotonin and higher amount of ligand-receptor complex formation (Pollier *et al.*, 2000). For these reasons the discovery of new SERT modulators is the focus of a number of studies with the aim of controlling symptoms of pathologies associated with altered serotonin levels (Warden *et al.*, 2008, MacGillivray *et al.*, 2011).

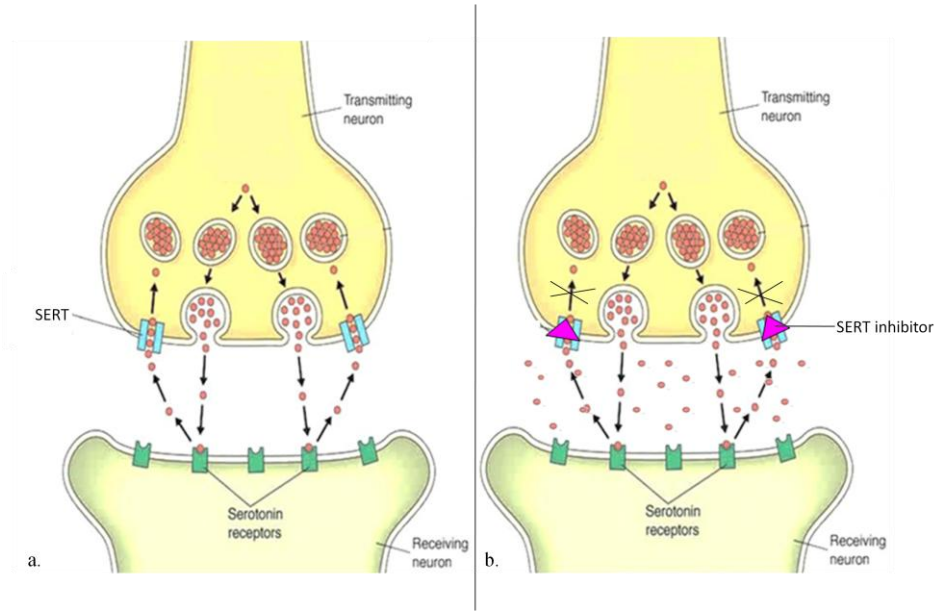


Figure 5-4. Scheme of the role of the serotonin reuptake transporter in the intracellular/extracellular serotonin balance. a. SERT reuptake serotonin in the releasing cell for it to be recycled. b. When a selective inhibitor of SERT is present, SERT is blocked and unable to reuptake serotonin effectively, resulting in increased amount of serotonin present in the synaptic space and subsequently more receptor-ligand complex formation.

An example of drug acting *via* SERT inhibition is imipramine. Imipramine is widely used in the fight against depression and anxiety (Russell *et al.*, 2001). Among the tricyclic antidepressants imipramine has the second highest affinity for SERT, after clomipramine. Studies have linked SERT modulation to appetite suppression and have indicated SERT as a potentially valid target for the treatment of obesity and food-linked dysfunctions (de Magalhaes-Nunes *et al.*, 2007, Talbot *et al.*, 2010). Imipramine is also used to control gastrointestinal dysfunctions (Talley et al., 2008, Abdul-Baki et al., 2009b). Studies have shown that low doses of imipramine are effective in reducing the symptoms of irritable bowel syndrome. For example, one study assessed the effect of imipramine on 56 patients

suffering from the syndrome, observing that 80.6% of the patients showed significant improvement of the symptoms after 12 weeks of treatment with 25 mg/ day of the drug (Abdul-Baki *et al.*, 2009a). However, using imipramine to control irritable bowel syndrome commonly causes a series of side effects, such as dizziness and extreme fatigue (Mavissakalian and Perel, 2000). The main limitation of the currently existing medications for irritable bowel syndrome is that they can only alleviate the discomfort caused by it but cannot completely eliminate the symptoms, nor cure the dysfunction (Mavissakalian *et al.*, 2002). A number of studies currently focus on finding new treatments for irritable bowel syndrome (Labus, 2007).

5.3.2. Green tea role in human health

Green tea is the most consumed beverage in the world, after water (Cabrera *et al.*, 2006a). Both whole extracts and individual components are long known for their health beneficial effects. Studies associate regular green tea intake with a wide range of benefits for human health, such as chemopreventive effects (Gao *et al.*, 1994, Cooper *et al.*, 2005, Lee, 2007), antiviral activity (Song *et al.*, 2005) and diabetes prevention (Waltner-Law *et al.*, 2002).
Green tea is a rich source of the polyphenols, catechins and epicatechins (fig. 5-5).

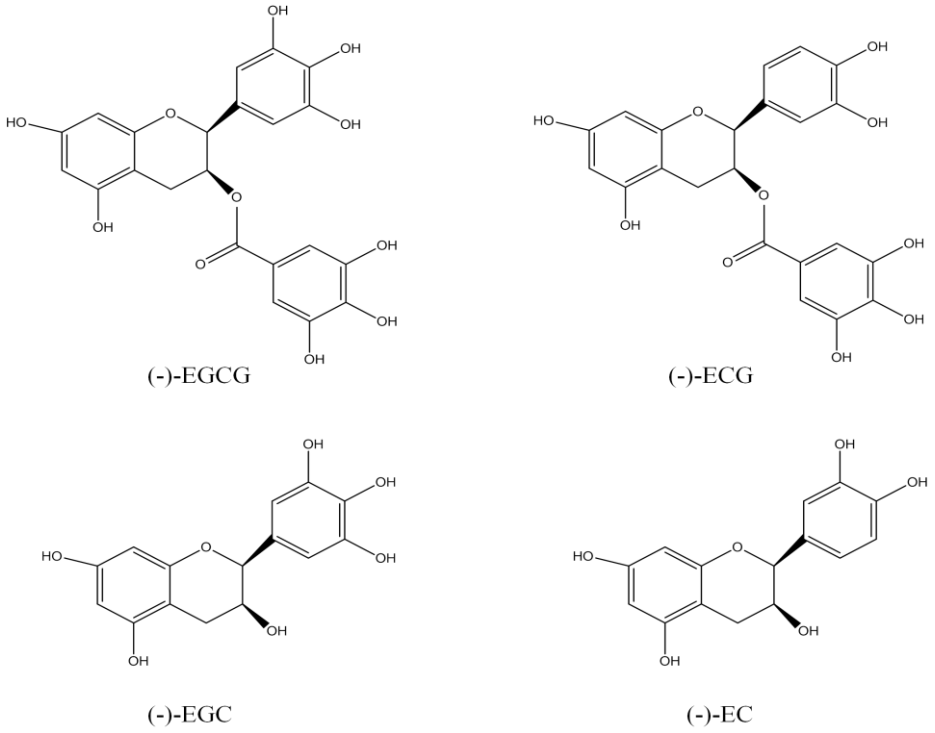


Figure 5-5. Chemical structure of the most abundant polyphenols present in green tea: epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC).

The relative amounts of bioactive components in green tea may vary, according to the origin of the leaves and the processing conditions. An average commercially-available green tea blend contains 3-10 times more catechins than black tea (Henning *et al.*, 2003), with catechins accounting for 60-80 % of the total polyphenol content (Cabrera *et al.*, 2006b). Green tea components have been associated with weight loss, enhancing daily energy expenditure and fat oxidation (Dulloo *et al.*, 1999). Epigallocatechin gallate (EGCG) from green tea has also been associated with weight loss. Animal studies showed that rats daily injected with EGCG for one week consumed up to 60 % less food (Kao *et al.*, 2000) compared to control.

Further studies suggested that green tea itself is a potent modulator of appetite and facilitates weight loss, by enhancing fat burning and increasing the turnover of serotonin and dopamine (Mirza *et al.*, 2013). In order to achieve significant effects on human health the recommended dose is at least 2-3 cups of the beverage per day (equivalent to 240-320 mg catechins). The maximum recommended amount is 10 cups per day (Fujiki *et al.*, 2002). Despite the known health beneficial effects, green tea is still very little consumed in Western countries (Graham, 1992) and often supplements are used as a replacement or in addition to beverage consumption.

In the present study whole extracts and individual green tea compounds were used as potential modulators of SERT, using the Caco-2 cell model. Caco-2 cells were used as they constitutively express the human SERT (Martel et al., 2003b).

The table below shows the composition of the green tea blend used in the present work (table 5-1).

Table 5-1. Green tea composition. Values are given as compound percentage in the extracts. The values result from unpublished carried out previously carried out in the research group.

Green tea compound	%
(-)- epigallocatechin gallate	17.83
(-)- epigallocatechin	12.39
(-)- epicatechin gallate	3.15
(-)- epicatechin	2.2
(+)- catechin	1.62
(+)- gallocatechin	9.45
(-)- catechin gallate	0.15
(-)- gallocatechin gallate	0.14

5.3.3. Coffee and role in human health

Coffee is also long known for its beneficial effects on human health (van Dam, 2008, George et al., 2008, Shin et al., 2010). However, a number of side effects have been associated with high coffee consumption, mainly ascribable to caffeine (Leviton and Cowan, 2002, Cornelis and El-Sohemy, 2007). The increasing availability of decaffeinated beverages raises the question of whether the positive effects derived from coffee are caffeine-dependent and whether caffeine-free products would still result in improved health (Skerrett, 2012).

To date, no studies have reported the effect of coffee and coffee components on serotonin balance within the gut. In the present work the effect of whole coffee extracts and individual components was assessed on SERT, expressed in Caco-2 cells.

5.4. Results

5.4.1. Experimental design validation

Validation of the method was carried out. Expression of the SERT gene (*SLC6A4*) was evaluated in Caco-2 cells through RT-PCR. Liver HepG2 cells were used as comparative cell model to evaluate *SLC6A4* expression. Serotonin uptake was also evaluated in the cell model, assessing concentration- and time-dependency of the transport, using tritium-labelled serotonin. Finally Sodium-dependency of serotonin uptake was evaluated, comparing uptake between sodium-containing medium and sodium-depleted medium. Details on the methods used are given in chapter 2.

When 100 ng cDNA were used, Ct value in Caco-2 was found to be 28 for *SLC6A* and 21 for the housekeeping gene, GAPDH. In HepG2 cells, when 100 ng cDNA were used, the Ct value was found to be 32 for *SLC6A4* and 18 for GAPDH (fig. 2-2).

Serotonin uptake proved to be linear for the time interval considered: 1-15 min (fig. 5-6). Concentration-dependent uptake was also observed for the range of concentrations used, 0.1-2.5 µM (fig. 5-7) and the Km calculated to be 0.9 µM.

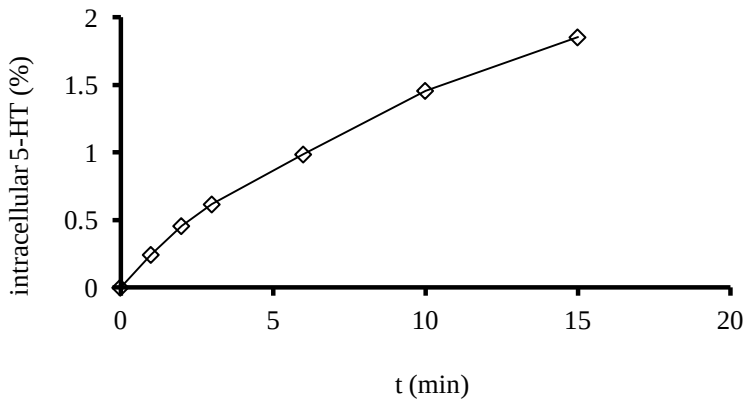


Figure 5-6. Time-dependent uptake of serotonin in Caco-2 cells. Cells were incubated with 0.2 µM tritium-labelled serotonin for 1-15 min at 37 °C. Cells were lysed and the content of each well collected separately, to determine the intracellular serotonin in each well. The amount of serotonin taken up in each well was measured based on the β emissions of tritium using a scintillation counter, as counts per minute (CPM) and converted into decays per minute (DPM). For each incubation time, the percentage of uptake is reported (e.g. the graph shows that after an incubation of 10 minutes almost 1.5 % of the initial 0.2 µM serotonin is taken up). Representative results are shown.

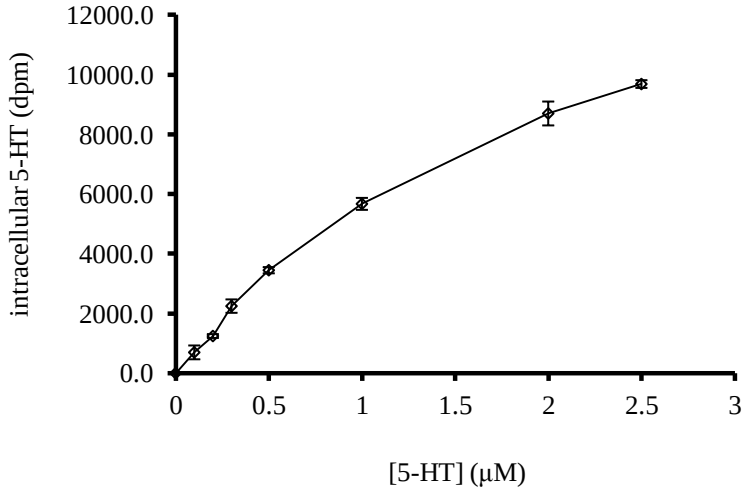


Figure 5-7. Concentration-dependent uptake of serotonin in Caco-2 cells. Cells were incubated for 6 min with 0.1-2.5 μM tritium-labelled serotonin. Intracellular uptake was measured based on the β emissions of tritium using a scintillation counter. Results shown represent average of triplicates ± St. Dev.

Among the preliminary experiments, sodium-dependence of serotonin uptake was assessed. When serotonin uptake was carried out in sodium-free medium, uptake was decreased by 40 % (fig 5-8).

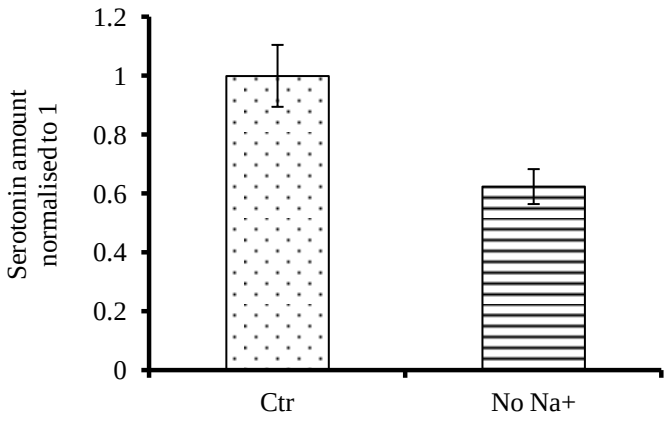
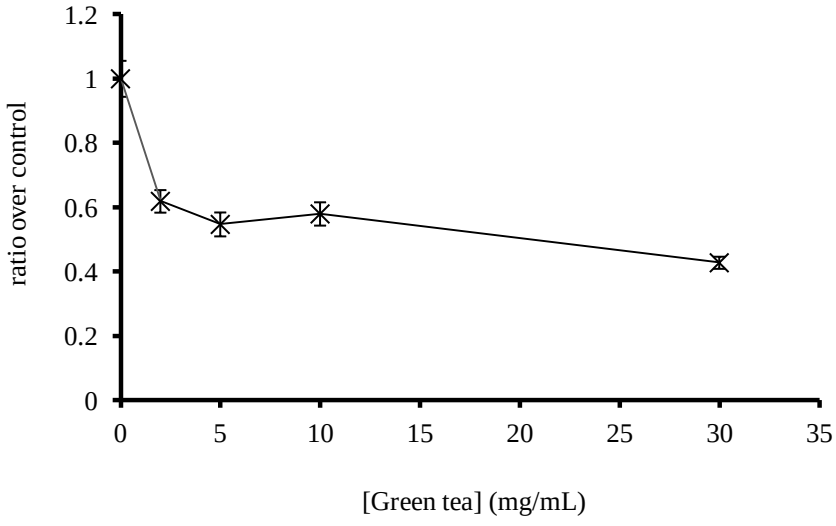


Figure 5-8. Sodium-dependent uptake of serotonin in Caco-2 cells. Cells were incubated for 6 minutes with 0.2 μM tritium-labelled serotonin in either standard transport medium (containing sodium, Na^+) or sodium-depleted medium (where sodium had been replaced with potassium, K^+). Intracellular serotonin was measured based on the β emissions of tritium using a scintillation counter. The intracellular serotonin amount was normalized to 1. Results represent the average of triplicates ± St. Dev.

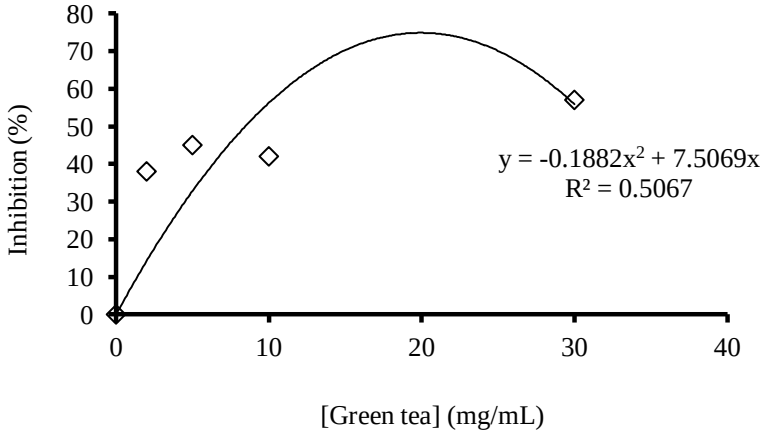
5.4.2. Modulation of SERT using whole green tea extracts and individual compounds at physiological concentrations

Whole green tea extracts were tested on Caco-2 cells at concentrations between 2 and 30 mg/mL as potential modulators of SERT. Green tea inhibited serotonin uptake in a concentration-dependent manner (fig. 5-9). IC_{50} was calculated resulting to be 8.45 mg/mL. At physiological concentrations ≤ 1 mM, none of the tested concentrations of EGCG affected serotonin uptake. EGC, ECG and EC inhibited uptake in a concentration-dependent way. EGC triggered a 9, 18 and 21 % inhibition of the serotonin uptake at 0.1, 0.5 and 1

mM respectively. ECG inhibited uptake at 0.5 and 1 mM by 12 and 16 % respectively. The effect of EC was only significant at 1 mM, when a 15 % inhibition was observed (fig. 5-10).



a.

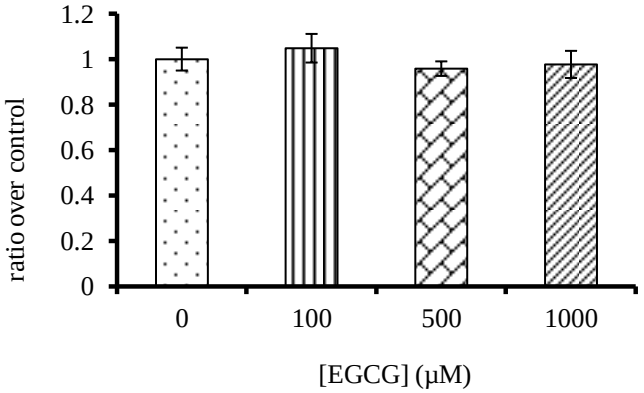


b.

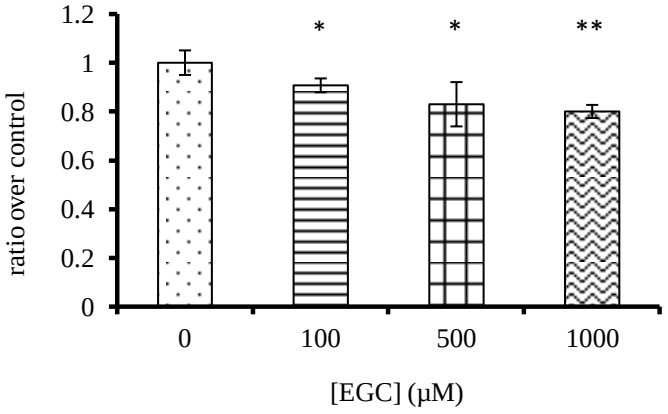
Figure 5-9. Effect of whole green tea extracts on serotonin uptake. (a) Whole extracts were prepared at concentrations between 2 and 30 mg/mL and co-incubated for 6 minutes with tritium-labeled serotonin. The intracellular amount of serotonin was calculated on the basis of the β emissions of tritium, using a scintillation counter.

Results are expressed as the ratio of the intracellular serotonin from cells treated with green tea over control cells, untreated. Results represent average of triplicates ± St. Dev.

(b) The IC_{50} was calculated by plotting the percentage of inhibition against the corresponding green tea concentration (mg/mL) used. The predicted polynomial was used, where x = IC_{50}.



a.



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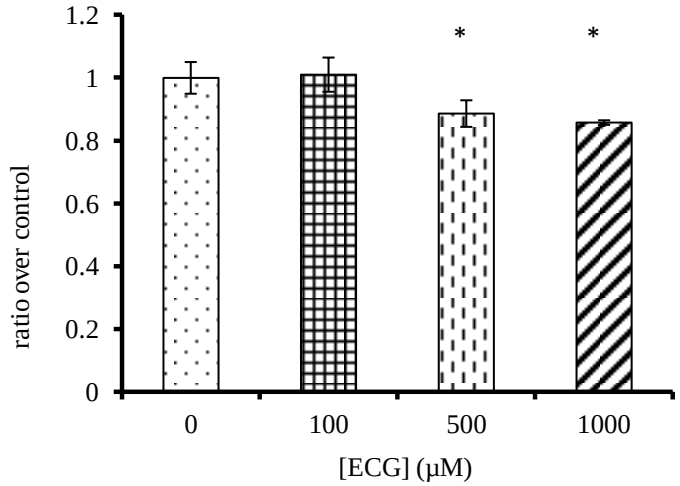
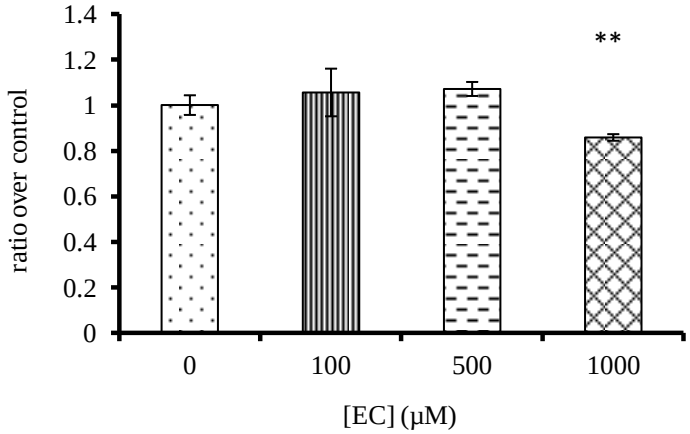


c.



d.

Figure 5-10. Effect of physiological concentrations of individual green tea components on serotonin uptake in Caco-2 cells. Cells were co-incubated for 6 min at 37 °C with tritium-labelled serotonin and EGCG (a), EGC (b), ECG (c) or EC (d) at concentrations ≤ 1 mM. Intracellular levels of serotonin were measured using a scintillation counter, based on the β emissions of tritium. Results are expressed as the ratio of intracellular serotonin from cells treated with green tea components over control cells, untreated.

Results represent the average of triplicates ± St. Dev.

* = $P < 0.05$ and ** = $P \leq 0.01$.

5.4.3. Modulation of SERT using supplement concentrations of green tea

Supplement concentrations equivalent to 7 cups of green tea were used as modulators of serotonin uptake. Concentrations of 11.6 mM EGCG, 12 mM EGC, 2.15 mM ECG and 2.4 mM EC were used, resulting in decrease in intracellular serotonin of 30, 54, 43 and 23 % respectively (fig 5-11).

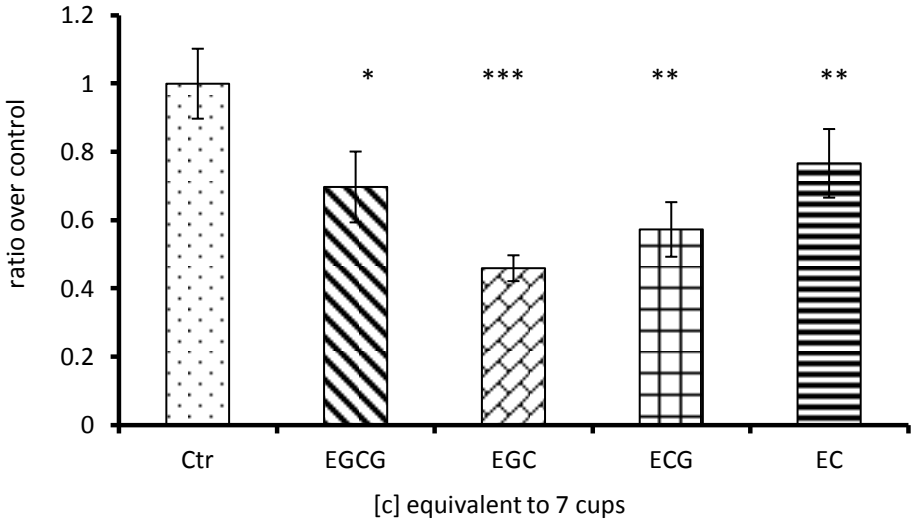


Figure 5-11. Effect of supplement concentrations of green tea components on serotonin uptake in Caco-2 cells. Cells were incubated with EGCG, EGC, ECG and EC at 11.6, 12, 2.15 and 2.4 mM respectively, corresponding to 7 cups of green tea.

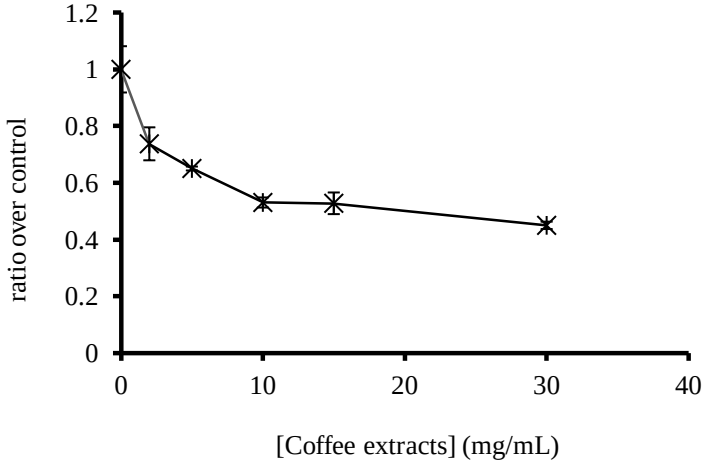
Results are expressed as ratio of the intracellular serotonin in cells treated with the green tea compounds over untreated cells.

Results represent the average of triplicates ± St. Dev.

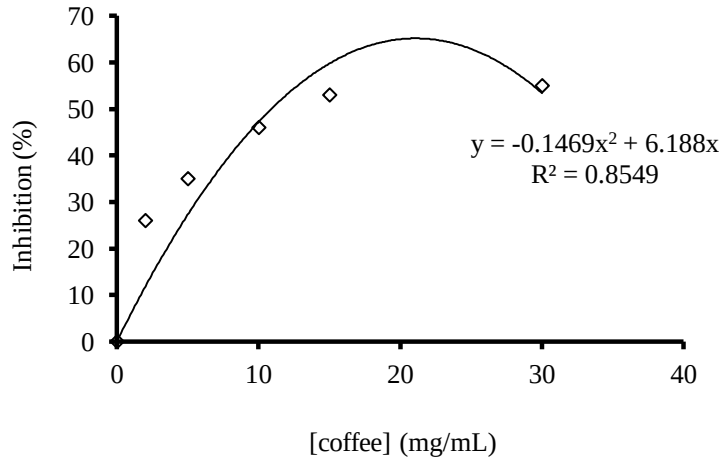
* = $P < 0.05$, ** = $P \leq 0.01$ and *** = $P \leq 0.001$.

5.4.4. Inhibition of 5-HT uptake using whole coffee extracts

Whole coffee extracts were initially tested. They showed to inhibit 5-HT uptake in a concentration-dependent manner (fig. 5-12). The IC_{50} for coffee extracts was calculated to be 10.9 mg/mL.



a.



b.

Figure 5-12. Coffee inhibition of serotonin uptake. (a) Whole extracts were prepared at concentrations between 2 and 30 mg/mL and co-incubated for 6 minutes with tritium-labeled serotonin. Results are expressed as the ration of intracellular serotonin from cells

treated with coffee over control cells, untreated. Results represent the average of triplicates ± St. Dev.

(b) The IC_{50} was calculated by plotting the percentage of inhibition against the corresponding coffee concentration (mg/mL) used. The predicted polynomial was used, where x = IC_{50}.

Individual coffee compounds were selected for testing, starting with caffeine. Caffeine was tested at 2.3 mM, a concentration equivalent to 15 mg/mL coffee (strong coffee). At this concentration, no effect on serotonin uptake was observed (fig. 5-13).

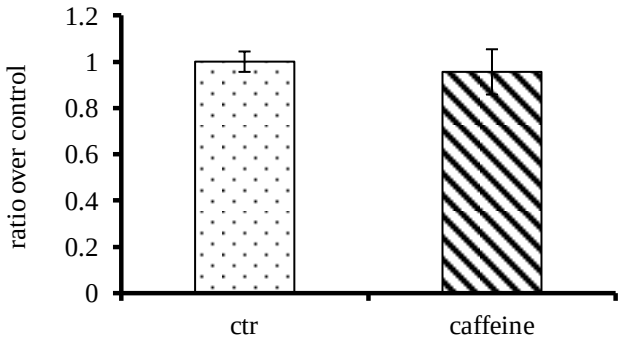


Figure 5-13. Effect of caffeine on serotonin uptake in Caco-2 cells. Cells were incubated for 6 min at 37 °C with 2.3 mM caffeine and tritium-labelled serotonin. Intracellular serotonin was measured using a scintillation counter, on the basis of the β emissions of tritium. Results are expressed as ratio of intracellular serotonin in cells treated with caffeine over control cells, untreated. Results represent the average of triplicates ± St. Dev.

5-CQA and 3-CQA are chlorogenic acids, they are among the most abundant compounds in the selected coffee blend (table 5-2).

Table 5-2. Coffee composition. Values are given as mg of compound per grams of coffee powder (Hoelzl *et al.*, 2010).

Coffee compound	Amount (mg/g solid)
5-caffeoylquinic acid	41
4-caffeoylquinic acid	12.4
3-caffeoylquinic acid	10.5
5-feruoylquinic acid	7.9
4,5-di-caffeoylquinic acid	5.8
3,4-di-caffeoylquinic acid	5.7
3,5-di-caffeoylquinic acid	4
4-feruoylquinic acid	2.2
caffeic acid	0.279
Ferulic acid	0.077
3,4-dimethoxycinnamic acid	0.037
caffeine	29

3-CQA and 5-CQA were tested at 3.4 and 0.9 mM respectively, corresponding to 15 mg/mL coffee. 3-CQA inhibited serotonin uptake by 4 %, while 5-CQA had no effect (see fig. 5-16).

3,4-diCQA, 3,5diCQA, CA, FA, 3,4-DMCIN and 5-FQA were tested at physiological concentrations $\leq$ 3 mM. 3,4-diCQA and 3,5-diCQA had no effect (fig. 5-15). CA, FA, 5-FQA and 3,4-DMCIN acted as concentration-dependent inhibitors (fig. 5-14-fig. 5-16). The IC_{50} values for FA and 5-FQA were calculated to be 3 and 1.6 mM respectively.

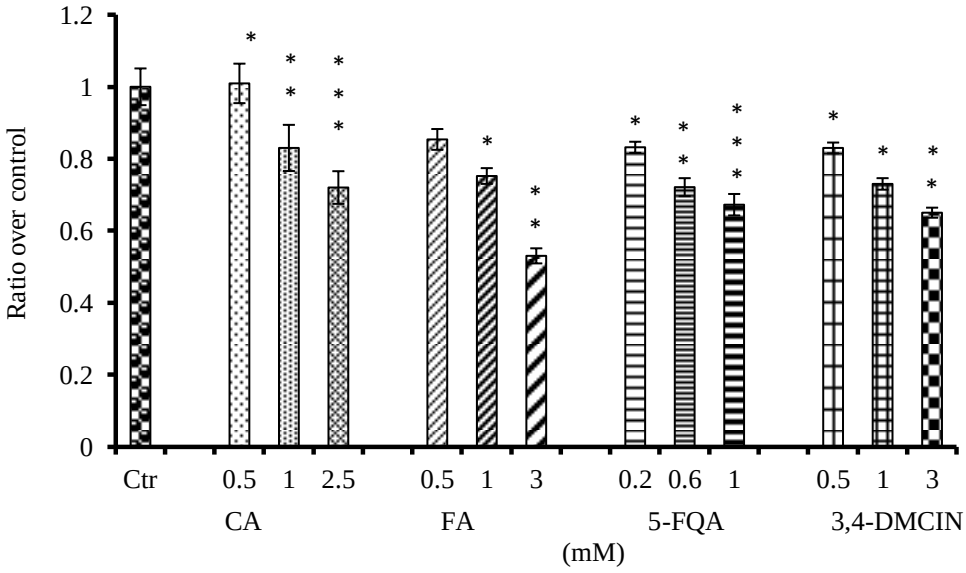


Figure 5-14. Effect of physiological concentrations of individual coffee components on serotonin uptake. Cells were incubated at for 6 min at 37 °C with physiological concentrations ≤ 3 mM of CA, FA, 5-FQA or 3,4-DMCIN and 0.2 µM tritium-labelled. Intracellular serotonin was measured based on the β emissions of tritium using a scintillation counter. Results are expressed as ratio of the intracellular serotonin from treated cells over untreated cells.

Results represent the average of triplicates ± St. Dev.

* = $P < 0.05$, ** = $P \leq 0.01$ and *** = $P \leq 0.001$.

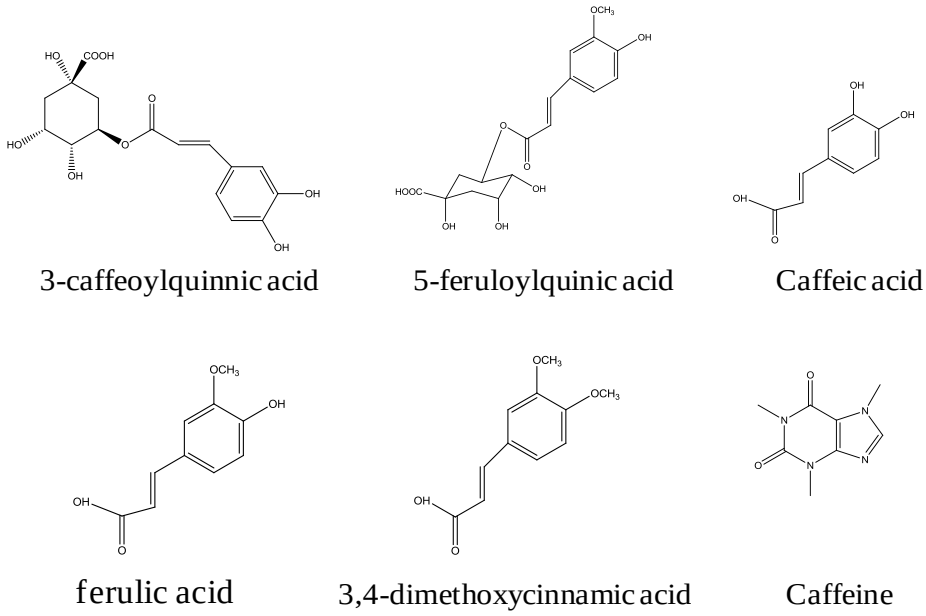


Figure 5-15. Chemical structures of bioactive compounds and caffeine from coffee tested for ability to inhibit SERT.

Having identified potential inhibitors, the predicted inhibition values for the active compounds were calculated for concentrations equivalent to 15 mg/mL coffee. CA (23 µM), FA (6 µM), 3,4-DMCIN (2.7 µM) and 5-FQA (0.4 mM) would inhibit 5-HT uptake by 0.4 %, 0.15 %, 0.0 7 % and 26 % respectively. Subsequently the predicted inhibitory effects were individually validated in the cell system. Results were in agreement with the predicted values, showing 26 % inhibition for 0.4 mM 5-FQA and no observable effect for the other compounds (fig. 5-16). Imipramine was used as positive control as a known down-regulator of SERT. 75 % inhibition was observed when treating cells with 5 µM imipramine (fig. 5-16).

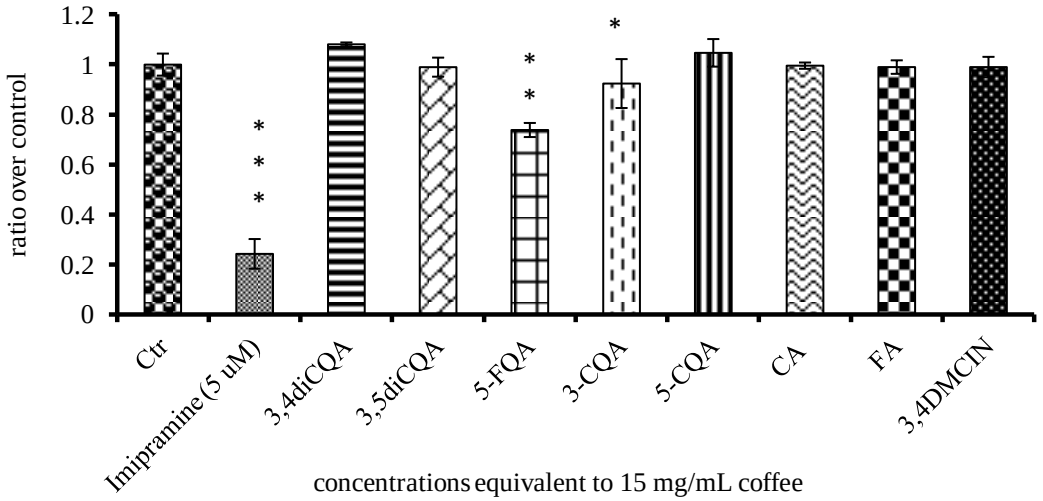


Figure 5-16. Effect of selected coffee components on serotonin uptake in Caco-2 cells. Cells were incubated for 6 min with 0.2 µM tritium-labelled serotonin and caffeine, 3,4-diCQA, 3,5-diCQA, 5-FQA, 3-CQA, 5-CQA, CA, FA or 3,4-DMCIN at concentrations equivalent to 15 mg/mL coffee. Intracellular serotonin was measured based on the β emissions of tritium using a scintillation counter. Results are expressed as ratio of intracellular serotonin from treated cells over control cells, untreated.

Results represent the average of triplicates ± St. Dev.

* = $P < 0.05$, ** = $P \leq 0.01$ and *** = $P \leq 0.001$.

These results highlight the difference between absolute and relative potency of the tested compounds. When considering 1 mM of 5-FQA, FA, CA and 3,4-DMCIN uptake was inhibited by 34, 25, 17.4 and 26 % respectively.

Whole coffee extracts tested at a concentration of 15 mg/mL inhibited 5-HT uptake by 53 %. Overall, individual coffee compounds accounted for 32 % inhibition, over half of the observable inhibition (fig. 5-17 and table 5-3).

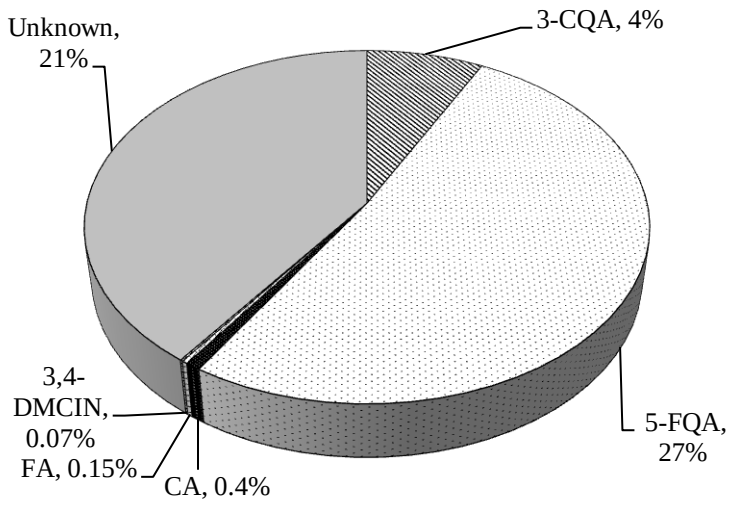


Figure 5-17. Contribution of individual coffee compounds to the observed inhibition of serotonin uptake in Caco-2 cells. Over half of the observable inhibition triggered by coffee was characterized and associated with specific compounds. 5-FQA alone accounted for 49 % of the inhibitory effect.

Table 5-3. Absolute and relative potency of individual coffee compounds on serotonin uptake. Coffee components are listed in order of their abundance in the coffee blend. Absolute potency of each compound is reported at a concentration of 1 mM. The contribution to the overall inhibition is shown at concentrations equivalent to 15 mg/mL coffee.

Compound	Amount in coffee (mg/g solid)	Inhibition by 1 mM (%)	Inhibition by concentrations equivalent to 15 mg coffee/ mL (%)
5-CQA	40.6	0	0
3-CQA	10.5	9	4
5-FQA	7.9	34	26
3,4-diCQA	5.7	0	0
3,5-diCQA	4	0	0
CA	0.279	17.4	0.4
FA	0.077	25	0.15
3,4-DMCIN	0.037	26	0.07
Caffeine	29	0	0

FA and 5-FQA were tested at physiological concentrations (1-3 mM and 0.5-2.5 mM respectively), co-incubating with a range of serotonin concentrations (0.05-1 μM). The aim was to assess whether FA and 5-FQA acted as competitive inhibitors of serotonin uptake. Both compounds were shown to be competitive inhibitors (fig. 5-18 and 5-19).

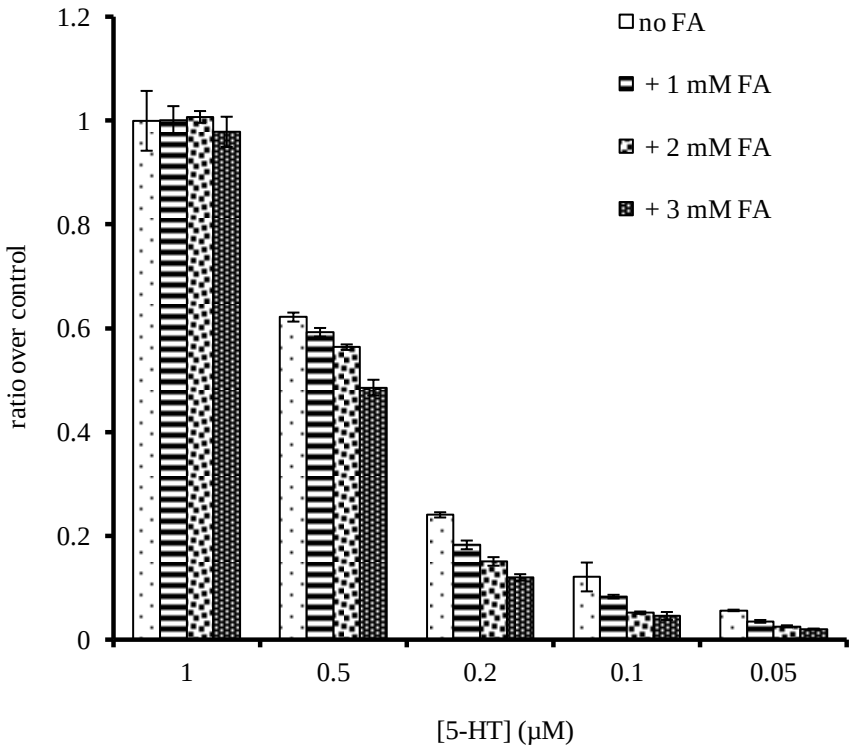


Figure 5-18. Competitive inhibition of ferulic acid on serotonin uptake in Caco-2 cells. Cells were incubated with a range of concentrations of FA in combination with a range of tritium-labelled serotonin concentrations. Intracellular 5-HT was measured based on the β emissions of tritium using a scintillation counter. Results are expressed as ratio of the intracellular 5-HT from treated cells over untreated cells.

The results represent the average of triplicates ± St. Dev.

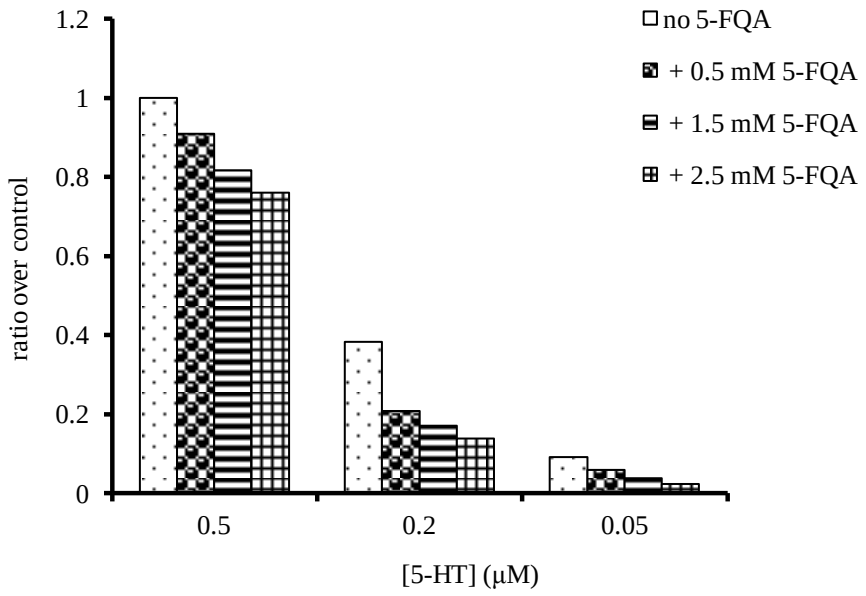
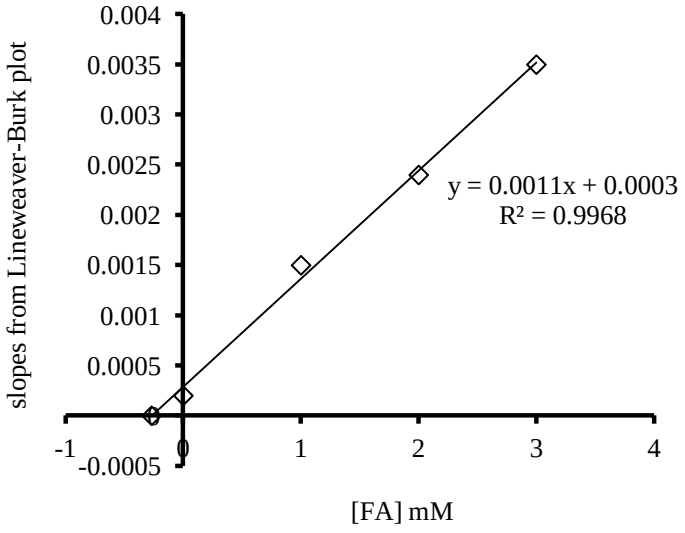
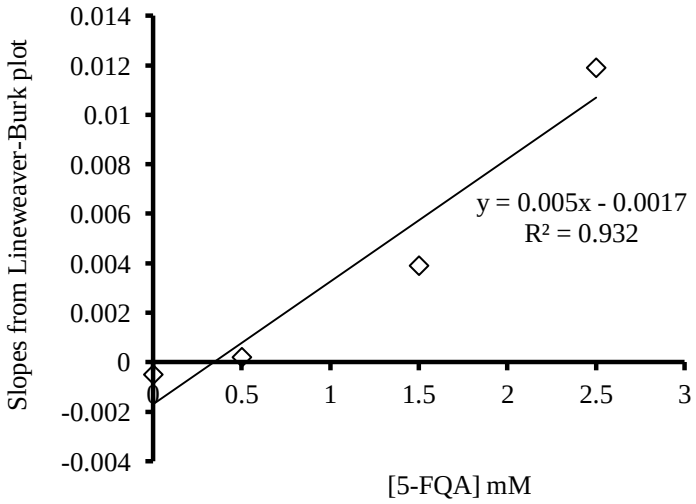


Figure 5-19. Competitive inhibition of 5-*O*-feruoylquinic acid on serotonin uptake in Caco-2 cells. Cells were incubated with a range of concentrations of 5-FQA in combination with a range of tritium-labelled serotonin concentrations. Intracellular 5-HT was measured based on the β emissions of tritium using a scintillation counter. Results are expressed as ratio of the intracellular 5-HT from treated cells over untreated cells. Typical results are shown.

The inhibition constants (K_i) for FA and 5-FQA were calculated using the Dixon plot and resulted to be 270 µM and 340 µM respectively (fig. 5-20).



a.



b.

Figure 5-20. Calculation of the inhibition constants (K_i) for ferulic acid and 5-*O*-feruoylquinic acid, using the Dixon plot. The K_i was calculated for FA (a) and 5-FQA (b) using the Dixon plot. In the equation $x = K_i$

Hydrolysis of whole coffee extracts with the enzyme chlorogenate esterase was carried out, in order to assess if and in which measure chlorogenic acids contributed to inhibit serotonin uptake. HPLC analysis showed that 84 % of the total chlorogenic acid content was hydrolysed after treatment with the enzyme (fig. 5-21). When using hydrolysed coffee for cell treatment, the inhibition potency of the whole coffee extracts drastically decreased, compared to non-hydrolysed coffee. Serotonin uptake was inhibited by 28 % and 47 % using hydrolysed and non-hydrolysed coffee respectively (fig. 5-22).

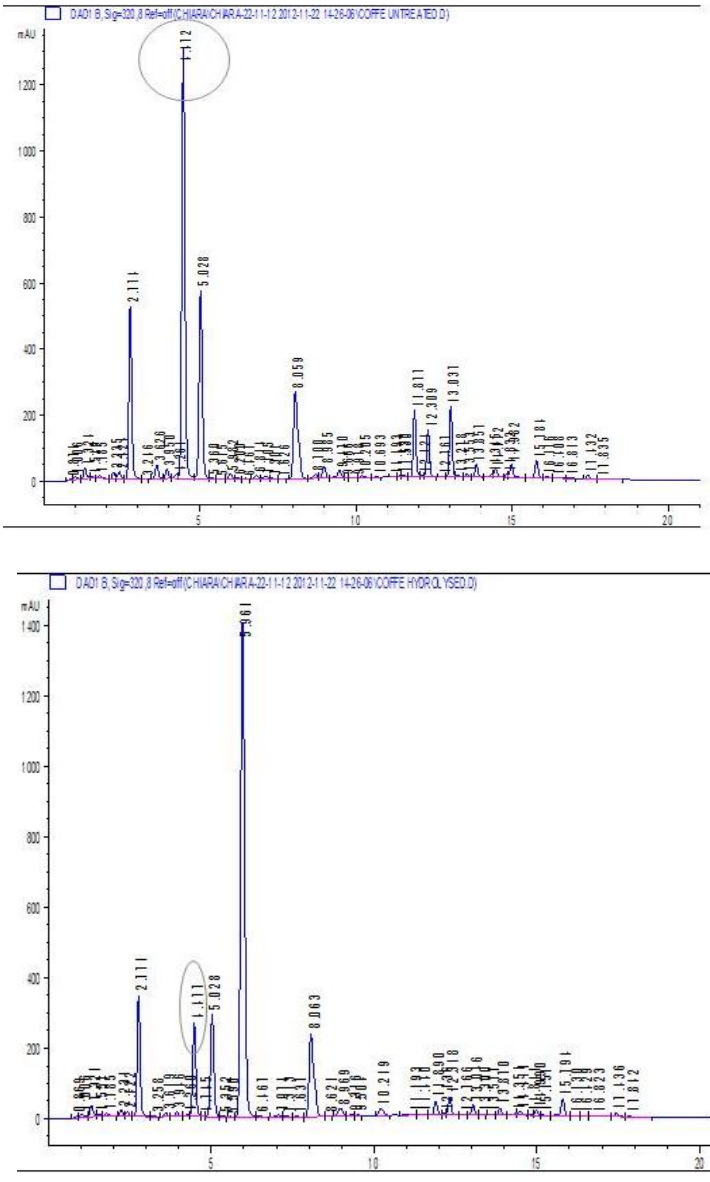


Figure 5-21. HPLC analysis of coffee hydrolysed with chlorogenate esterase. Whole coffee extracts, prepared using 15 mg soluble coffee/ mL water, were incubated for 30 min at

37 °C with the enzyme chlorogenate esterase. Hydrolysed samples (b) were analysed through HPLC and compared to control non-hydrolysed extracts (a). Analysis was carried out based on the retention time for chlorogenic acid, which resulted to be 1.11 minute, under the method conditions. Typical chromatograms are shown.

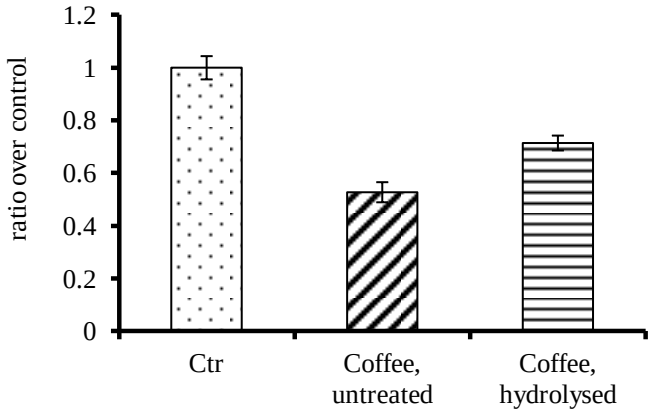


Figure 5-22. Effect of hydrolysed and non-hydrolysed coffee on serotonin uptake. Caco-2 cells were incubate for 6 min at 37 °C with 15 mg/mL hydrolysed or non-hydrolysed coffee extracts and 0.2 µM tritium-labelled serotonin. Intracellular serotonin was measured based on the β emissions of tritium using a scintillation counter. Results are expressed as ratio of the intracellular serotonin from cells treated with hydrolysed coffee over serotonin from control cells, treated with non-hydrolysed coffee extracts.

Results represent the average of triplicates ± St. Dev.

5.5. Discussion

5.5.1. Understanding the mechanisms of serotonin uptake in intestinal cells

Multiple mechanisms exist to re-uptake serotonin in the intestinal cells (Freeman *et al.*, 2004). It is known that one of the ways serotonin is taken back up in the releasing cells is through SERT, a sodium-dependent protein (Huot et al., 2011). When serotonin transport was carried out in sodium-free medium, uptake was dramatically decreased. This proved the determinant role of the sodium-dependent SERT and highlighting, according to expectations, the existence of multiple mechanisms responsible for 5-HT uptake. The results

also showed that Caco-2 cells are a representative model for intestinal cells, displaying molecular pathways comparable to *in vivo* intestine cells. Serotonin imbalance in the gut is associated with a range of pathologies, such as irritable bowel syndrome. So far no treatments exist capable of completely eliminate the symptoms and discomfort coming from this syndrome. Also, the existing drugs used often cause a range of unpleasant side effects. The current work showed that selected polyphenols, from coffee and green tea, acted as modulators of serotonin uptake, inhibiting reuptake through SERT. The possibility of using food compounds to treat irritable bowel syndrome and other gut dysfunctions associated with serotonin imbalance is extremely interesting. In fact, if natural products could be used to treat these pathologies, it would be possible to eliminate the side effects, caused by long-term treatment with synthetic drugs, such as imipramine.

5.5.2. Effect of green tea on serotonin uptake

Studies have associated green tea consumption with appetite suppression and weight loss, *via* a range of mechanisms including alteration of serotonin levels (Hursel *et al.*, 2009). However only a limited number of studies have investigated the molecular mechanisms involved in green tea effects on appetite suppression and weight loss (Jurgens *et al.*, 2012). No studies so far exist on the role of green tea on serotonin re-uptake *via* SERT.

5.5.2.1. Effect of green tea whole extracts and individual components tested at physiological concentrations

The present work reports for the first time a role for green tea whole extracts and individual components in inhibiting serotonin re-uptake *via* SERT. The intestinal model Caco-2 cells

were used, as they constitutively express SERT (Martel et al., 2003a). Whole green tea extracts showed concentration-dependent inhibition of serotonin uptake. Concentrations as low as 2 mg/mL, corresponding to a weak cup of green tea, triggered serotonin uptake inhibition. Physiological concentrations ≤ 1 mM of individual green tea components (EGCG, EGC, ECG and EC) showed to have a limited effect on serotonin re-uptake. The compound triggering highest inhibition of serotonin uptake was EGC, which displayed to inhibit uptake in a concentration-dependent manner at all the tested concentrations. EGC inhibited uptake up to 21 % (when tested at 1 mM). Interestingly EGCG was the least effective inhibitor. In fact none of the tested physiological concentrations of EGCG produced any effect on serotonin uptake. It is noteworthy that EGCG was the most abundant component in the green tea blend used for the work, while EGC was the second most abundant compound. The results obtained testing selected green tea components showed that initial abundance of certain compounds in the green tea blend did not necessarily correlate with a high effect of the compound on biological processes, such as serotonin uptake. An important step to do would be to test other green tea components, present in green tea at small levels. They could be tested both individually and in combination with each other, to assess whether they have any effect on serotonin uptake.

It would also be interesting to assess whether the four compounds initially selected (EGCG, EGC, ECG and EC) could trigger more/less inhibition of serotonin uptake when tested in combination with each other.

5.5.2.2. Effect of individual green tea components tested at supplement concentrations

Supplement concentrations of the individual green tea compounds EGCG, EGC, ECG and EC were subsequently tested. Concentrations equivalent to 7 cups of green tea used. According to published literature 10 cups of green tea per day is the maximum recommended intake (Halegoua-De Marzio *et al.*, 2012), thus 7 cups would be a safe amount. Studies have shown that, despite the limited bioavailability of polyphenols, local concentration of these compounds in the colon can reach much higher concentrations compared to the systemic circulation. A study from Duthie *et al.* showed that after ingestion of 500 mg of polyphenols, the plasma concentration reached by these compounds was 50 μM. However, their concentration in the colon was 3 mM, 60 times higher than in the plasma (Duthie *et al.*, 1998). According to these findings, if 7 cups of green tea were drunk each day, they would provide the colon with polyphenol concentrations high enough to modulate serotonin uptake.

However the final concentrations in the colon would still be diluted compared to the initial intake, due to progressing through the gastrointestinal tract. An approach to overcome dilution could be designing drugs containing the equivalent amount of 7 cups of green tea. These drugs would have to be designed so that, once ingested, they would not be affected by movement through the gastrointestinal tract, but they would release their intact content once they reach the colon.

When using these concentrations, a high inhibition of serotonin re-uptake was observed, with consequent increase of the neurotransmitter availability in the extracellular space.

Among the tested green tea compounds, the most powerful effect on serotonin re-uptake was observed when incubating the cells with EGC, both at physiological and at supplement

concentrations. A correlation between EGCG supplement consumption and weight maintenance in patients who were previously obese has been reported (Westerterp-Plantenga *et al.*, 2005). In this study male and female volunteers were given 45 mg EGCG before meals and displayed reduced weight re-gain compared to the volunteers administered placebo tablets. The study did not investigate the mechanism of action of EGCG but supports evidence for the health beneficial effects of green tea components at supplement concentrations. It is interesting to note that, among the compounds tested at supplement concentrations in the present work, EGCG was the second least effective inhibitor of serotonin uptake, after EC. It would be interesting to assess the effect of EGC using human volunteers and compare the results obtained with those published from Westerterp-Plantega *et al*.

Other interesting results were observed in a study from 2007 (Nagao *et al.*, 2007). 240 obese patients were given green tea extracts, rich in catechins, over a period of 12 weeks. Volunteers displayed reduced hip circumference, body fat ratio and body mass index, compared to placebo treated patients.

Further studies could be designed to assess the biological repercussions of serotonin modulation, associated with incubation of the cells with individual green tea components. It would be also interesting to assess the effect of whole green tea extracts, tested on Caco-2 cells at supplement concentrations and compare serotonin uptake to uptake obtained incubating the cells with the same extracts selectively depleted of one component per time. This experimental design could clarify the contribution of each green tea components while assessing the overall effect of green tea extracts when individual compounds are allowed to interact with each other.

5.5.3. Effect of coffee on serotonin uptake

Coffee is long known to have beneficial effects on the human health (Harries-Rees, 2005, Taylor, 2008), although the mechanisms of actions are not fully known (Rogers *et al.*, 1995). Studies have associated a wide range of coffee effects to the presence of caffeine, an alkaloid compound acting on the central nervous system as stimulator (Chou and Benowitz, 1994). Consumption of caffeine-containing products has repercussions on the serotonin levels in various neurological ways, for example increasing the levels of the hormone cortisol (Griffiths and Chausmer, 2000, Cummings et al., 2013). Though not much is known about the effects of coffee on serotonin re-uptake within the gastrointestinal tract (Higdon and Frei, 2006). The present work reported a caffeine-independent effect of coffee on inhibition of intestinal serotonin re-uptake. Previous studies agree with these findings, having demonstrated that both caffeinated and decaffeinated drinks link to intestinal stimulation and increased bowel movements (Brown *et al.*, 1990, Rao *et al.*, 1998). These results could be crucial to develop health-promoting caffeine-free drinks. These drinks could be used as enhancers of the extracellular serotonin levels in target organs, such as the gut.

The inhibitory effect of whole coffee extracts and individual polyphenols on SERT activity were assessed for the first time in the present work.

Whole coffee extracts proved to inhibit uptake in a concentration-dependent manner. This result is in agreement with previously published results, showing that coffee administered to rats altered the serotonin levels found in the brain (Yamato et al., 2002). Several coffee components showed to inhibit serotonin uptake in a concentration-dependent manner, when tested at physiological concentration $\leq$ 3 mM. 1 mM of 5-FQA, FA, CA and 3,4-DMCIN inhibited uptake by 33, 25, 17.4 and 26 % respectively. Though the amounts of FA, CA and

3,4-DMCIN in coffee are many fold lower than 1 mM, therefore when assessing the effect of these compounds related to their effective concentrations, contribution to the overall serotonin inhibition resulted limited. When testing these compounds at concentrations equivalent to a strong cup of coffee (15 mg/mL) the majority of compounds did not show any effect on the uptake of serotonin. 5-FQA and 3-CQA were the only compounds to display significantly inhibition of SERT when tested at concentrations equivalent to a strong cup of coffee. They inhibited uptake by 4 and 26 % respectively. These results highlighted the difference between absolute and relative potency of the tested phenolic compounds.

Previous literature has reported that phenolic compounds can inhibit SERT-mediated serotonin uptake using a cell model (Ofir *et al.*, 2003). The liquorice components, glabridin, 4′-*O*-methylglabridin and glabrene, inhibited serotonin uptake by 60, 53 and 47 % respectively.

The drug imipramine, which is used to cure depression and gut dysfunctions, was used as positive control, for its known role as a SERT inhibitor. According to published literature (Ofir *et al.*, 2003), imipramine proved to be a potent modulator of SERT. Inhibition triggered by imipramine was compared to the inhibition triggered by whole coffee extracts. The data presented suggested that strong coffee modulated SERT with a potency comparable to the drug imipramine in the intestine model Caco-2 cells.

5.5.4. Role of chlorogenic acids on serotonin re-uptake inhibition

Chlorogenic acids are esters of hydroxycinnamic acids (caffeic acid, *p*-coumaric acid or ferulic acid) with (-)-quinic acid (Kwon *et al.*, 2010) . Multiple compounds belong to this family and their relative abundance varies in food (Dupas *et al.*, 2006). Coffee contains a range of chlorogenic acids (Moon *et al.*, 2009). The blend used in the present work was rich

in chlorogenic acids (table 5-2). However authentic standards were not available for all the compounds present in the blend and therefore they could not all be individually tested on the cells.

Treatment of coffee with the enzyme chlorogenate esterase was carried out in order to assess if and in which measure the total content of chlorogenic acids contributed to inhibit serotonin uptake. When using hydrolysed coffee for cell treatment, the inhibition potency drastically decreased, compared to non-hydrolysed coffee. This result highlighted a role for chlorogenic acids in the inhibition of serotonin re-uptake.

5.5.5. Work limitations and prospectives for future work

In the present work Caco-2 cells were used as a model for the human intestine. Despite literature showing that Ccao-2 cells are representative of the *in vivo* intestine (Hilgendorf et al., 2007b), cell lines in general offer a limited amount of information. *In vivo*, every tissue is part of an organ, which in turn is part of a system (Bassingthwaighte, 2002). The main limitation of using a cell model is that, in this case, the cells will be exposed to selected compounds/environments, while *in vivo* cells are simultaneously exposed to a much larger variety of compounds. *In vivo* human intestinal cells are simultaneously exposed to an enormous variety of compounds, from a multitude of dietary and non-dietary sources, while in the cell model one individual food/food compound is used on the cells. It is most likely that *in vivo* all the food compounds present in the gastrointestinal tract at a specific time interact with each other and with the cells, possibly resulting in a different physiological response compared to the cell model. It would be interesting to carry out a human study to compare the *in vitro* results with findings obtained using human volunteers.

More studies will have to be carried out to assess the biological repercussions of altered serotonin balance within intestinal cells and on the mechanisms through which coffee and coffee compounds interact with serotonin uptake, resulting in inhibition.

Nevertheless this study represents a significant step into better understating the effect of coffee on serotonin levels within the human gut, demonstrating that coffee and individual coffee compounds inhibit serotonin reuptake in a dose-dependent manner and that caffeine does not affect the process.

The present work showed that chlorogenic acids play a crucial role in inhibiting serotonin uptake in the Caco-2 cells. However it is not clear whether individual compounds are responsible for inhibiting uptake alone or if they interact with each other. It would be interesting to test these compounds individually in the cell model. It would also be interesting to test various compounds combined with each other, to assess whether the inhibition of serotonin uptake, which has not yet been fully characterised, is the result of single compounds acting independently on SERT or whether these compounds interact with each other to give the final inhibition.

The limitations of the present work and the prospectives for future work are further discussed in chapter 6.

5.6. Conclusions

The present work showed that coffee and green tea whole extracts and individual compounds acted as inhibitors of serotonin reuptake in the intestine model Caco-2 cells. Green tea individual compounds had limited effect at physiological concentrations, although their effect was high at supplement concentrations, equivalent to 7 cups of green tea. Among the individual coffee compounds tested, caffeine did not affect serotonin reuptake, while 5-FQA resulted to inhibit uptake in the most powerful way at concentrations

equivalent to a strong cup of coffee (26 % inhibition). It would be interesting to assess the effect of the identified inhibitors *in vivo.* If these compounds proved effective in inhibiting serotonin reupkae *in vivo,* they could be used to design drugs to treat serotonin-dependent gut dysfunctions such as irritable bowel syndrome.

Chapter 6

General discussion and suggestions for future work

6.1. Potential use of coffee and greet tea polyphenols to treat gut dysfunctions

The present study showed that selected coffee and green tea polyphenols acted as modulators of serotonin uptake in the intestinal model Caco-2 cells. This finding is interesting and should be further investigated since it highlights the possibility of treating serotonin-dependant gut dysfunctions, such as irritable bowel syndrome, using natural compounds from food. Further studies will have to be carried out to assess if and to what extend the selected polyphenols act as modulators of the serotonin reuptake transporter *in vivo*.

6.1. Polyphenol absorption in the gastrointestinal system and the role of transporters

Polyphenols have received increasing interest in the last few decades, for their properties as antioxidants (Reboul et al., 2007a).

They have high structural diversity and are found in all plants and certain fungi (Mahbub *et al.*, 2013). Animals, including humans, cannot synthesize polyphenols, thus the only route of entrance of these compounds in the human body is ingestion through the diet (Pereira *et al.*, 2013). Over 8,000 polyphenols have been identified so far (Perez-Ternero *et al.*, 2013), however polyphenols most often are found in plants as biologically unavailable glycosides or polymers, that need chemical modification in order to become available. Degradation occurs extensively in the intestine and gives origin to different products (Silberberg *et al.*, 2006). The nature of the products depends largely on the type of food initially ingested

(Lambert *et al.*, 2003), the initial amount of polyphenol present in the meal, the chemical structure of the latter (Johnson, 1998) and the composition of the surrounding food matrix ingested from the host. The latter is important because the chemical interactions between molecules does impact on the effect they have on the human health (Roura *et al.*, 2008, D'Agostino *et al.*, 2012). A crucial role is also played by the unique set of enzymes found in each individual (Larrosa *et al.*, 2006, D'Archivio *et al.*, 2010).

Each step of polyphenol metabolism within the human body is determinant for the resulting bioavailability. In the mouth, during mastication, polyphenols are partially released from the food matrix (Ginsburg *et al.*, 2013). They interact with salivary proteins, rich in the amino acid proline, either through hydrophobic interactions or hydrogen bonds (Ginsburg *et al.*, 2012). It is very interesting to note that different polyphenols have different affinity for the salivary proteins. Proanthocyanidins and flavanols interact the most with the salivary proteins (Obreque-Slier *et al.*, 2012). Great differences in affinity also exist among these two classes of polyphenols. For example, studies have shown that proanthocyanidin dimers, containing a bond between a C-4 and a C-8, had higher affinity for the salivary proteins than dimers containing a bond between a C-4 and a C-6. It was also shown that (+)-catechin interacted with the proline residues with much greater affinity than (-)-epicatechin (Spencer, 2003).

As a result of the binding between the salivary proteins and the polyphenols, soluble aggregates are formed. The described interaction is crucial for the resulting chemical activity of the proteins, since it impacts on the tertiary structure of the latter, modifying it due to the disruption of existing bonds and the formation of new ones (Bennick, 2002). Multiple protein-polyphenol complexes can join together resulting in large aggregates, which precipitate out of the solution (Soares et al., 2011). Interestingly it was observed that presence of these complexes did not impact greatly on the absorption and final availability of polyphenols (Tagliazucchi *et al.*, 2010). There is evidence that it is likely that a part of

the ingested polyphenols could be absorbed directly in the mouth. One example is the case of resveratrol (Walle *et al.*, 2004). However not many publications exist on this topic and nothing is known on the transporters which could be responsible of such movements across membranes. Buccal and gingival absorption has been proved for a variety of compounds and drugs and is currently used to deliver certain drugs (Darwish *et al.*, 2007, De Caro *et al.*, 2008, Sakata and Onishi, 2013). It would be interesting to carry out studies to identify such transporters. A first step would be to select simple foods, rich in polyphenols and assess the percentage of oral absorption for each of them, using human volunteers or using human gingival epithelium cell lines as an initial model (Calenic *et al.*, 2013).

Recent studies have shown that polyphenol exposure to saliva increases the oxidant scavenging activity (OSA) of these molecules and thus it highlights a further relevant role for mastication (Ginsburg *et al.*, 2012).

When food progresses through the gastrointestinal tract the compounds present in it, including the polyphenolic content, undergo dramatic changes, in terms of the pHs they are exposed to. The stomach is characterized by a high acidic environment, with pH that can be as low as 1-2 and that in normal healthy conditions does not exceed 4-5 (Fisher *et al.*, 1994). Individual studies were carried out using various compounds (malvidin-3-glucoside, resveratrol, catechins, proanthocyanidins) and demonstrated that polyphenols are incredibly tolerant to these pH changes. In contrast to what would be expected, they did not undergo any damage or structural change when exposed to different pHs (Lewandowska *et al.*, 2013).

Deglycosilation occurs in the small intestine, mainly *via* the activity of two endogenous *β*-glucosidase enzymes: the β-D-glucosidase and the lactase. *B*-D-glucosidase targets *β*1-4 bonds with broad substrate specificity, mainly acting on cleaving sugar complexes with glucose substitutes. Lactase is abundantly present at the brush board membrane of the enterocytes covering the intestinal *villi* and it is mainly involved in the breakdown of the

simple sugar lactose, found in milk, making possible its complete digestion (Teng *et al.*, 2012). After this step, the aglycones enter the epithelial cells through passive diffusion (Almon *et al.*, 2011). In the small intestine polyphenols undergo extensive glucuronidation, from uridine diphosphate glucuronosyltransferases (UGT). The UGTs catalyse covalent addition of glucuronic acid, recognised at the carboxyl-terminal end of the protein, to multiple lipophilic substrates and have a determinant role in detoxification by producing more polar compounds that are easily eliminated from the body. The amino-terminal half of these enzymes is the site of aglycone binding, thus its structure greatly changes between isoforms, while the carboxyl-terminal end is more conserved. Among the UGTs, UGT1A8 and UGT1A10 are the isoforms involved the most with glucuronidation of polyphenols, targeting the C5 and C7 of the A ring (Meech and MacKenzie, 1997). The human small intestine is also an important site for *O*-methylation and *O*-methylation of flavanol glucuronides (Daniel *et al.*, 2006). From the enterocytes, polyphenols are transported to the liver, which has the main role of increasing the overall solubility of compounds for their easier elimination from the body as xenobiotics (Forrester *et al.*, 1992, Wen and Walle, 2006). Thus polyphenolic compounds are mainly sulfated and methylated (Okushio *et al.*, 1999, De Santi *et al.*, 2000), resulting the almost complete conjugation of these compounds which, with exception of epicatechin gallate and epigallocatechin gallate, are found in blood as aglycones at extremely low concentrations (Selma *et al.*, 2009).

The main site of polyphenol absorption in the human body is the large intestine (Borges *et al.*, 2013). Polyphenols are initially modified by the intestinal microflora (Corona *et al.*, 2006) and then the products are absorbed, eliminated from the body or transported to the liver where they are transformed to monosulfates or monoglucuronides to be transported in the blood and subsequently excreted through the activity of the kidneys (Yang *et al.*, 2013).

In recent years, it has become evident that transporters play a major role in each of the metabolic steps of drug and xenobiotic transformation and transport across the various part

of the body. Polyphenols and their metabolites have gained attention for their promising health beneficial effects. It is known that they are highly absorbed, and therefore they must interact with transporters, due to the observation that after oral consumption of these compounds, they are all found into the blood circulation (Clifford *et al.*, 2013), although different absorption dynamics are expected according to the ingested compound(s). The relationship between polyphenols and transporters remains fascinating, both in terms of compound transport and in terms of transporter modulation from these compounds (Rodriguez-Proteau *et al.*, 2006). In fact many studies have been carried out assessing the effect that selected polyphenols have on specific transporters. However it is difficult to draw general conclusions, because the chemical structure of polyphenols varies considerably from one compound to another.

6.2. Limitations of *in vitro* studies

So far many studies have aimed to better understand the interactions between polyphenols and transporters. Though the majority of these studies are *in vitro* and only a limited number has been carried out both *in vitro* and *in vivo*, allowing a comparison of the results.

Chapter 4 highlights the differences existing between cell lines and *in vivo* cells. In fact, while chapter 3 showed that OAT3 is only significantly expressed in the human kidney, chapter 4 showed for the first time expression of the transporter in the liver cell line HepG2. This existing discrepancy further highlights the limitation of using cell lines. Although using cells is a powerful tool to model specific cellular activities, such as the mechanism of selected transporters, the use of cells does not offer a true representation of what happens *in vivo*. In a recent study, four polyphenols with different chemical structures were selected (emodin, chrysophanol, apigenin and resveratrol) to better understand their interaction with

transporters, both *in vitro* and *in vivo* (Teng *et al.*, 2012). The authors used the Caco-2 cell model for the *in vitro* assessment and blood samples from human donors for the *in vivo* assessment, identifying which transporters may be involved in the movement of the selected compounds. This study highlights the importance of using both *in vitro* and *in vivo* methods in order to obtain a more complete understating on the molecular mechanisms involved.

It is noteworthy that this study did not identify significant differences between the results obtained using the *in vitro* and the *in vivo* models.

A different example of such a comparison between *in vivo* and *in vitro* studies involved the assessment of the potential effect of orange juice polyphenols on decreasing the risk of cardiovascular disease (Vinson *et al.*, 2002). Interestingly this work highlights big differences between the results obtained using *in vitro* and *in vivo* measurements. A model of heart disease, based on measuring the inhibition of lower density lipoproteins promoted by cupric ion, and a hamster model of atherosclerosis were used for the *in vivo* and *in vitro* measurements respectively. The orange juice polyphenols showed promising protective effects *in vivo* but not *in vitro*.

In chapter 5, the effect of whole green tea and coffee was assessed, testing both whole extracts and individual compounds as down-regulators of the serotonin reuptake transporter. In the case of both whole extracts, time- and concentration-dependent down-regulation was observed. For certain individual coffee compounds it was shown that at physiological concentrations they were powerful down-regulators of SERT. In contrast, none of the tested individual compounds from green tea at physiological concentrations displayed a high modulatory effect on SERT. In both cases, experiments were carried out using a model, Caco-2 intestinal cells. There are multiple limitations in using such a cell model. First of all, when using cell lines, the cell model is created using cancerous cells. These cells show physiological and morphological differences compared to healthy cells and often present a very altered gene expression profile (Richmond and Su, 2008). Although experimental

technologies have been developed to assess the differences between immortalised cell lines and primary cells from the same tissue, these technologies are often extremely difficult to perform and only take into account specific aspects of the cell physiology (Tong *et al.*, 2009). For this purpose, bioinformatic tools could be extremely useful, since they are relatively cheap to use and give comprehensive information on the most different aspects of gene expression and cell physiology. For example, a bioinformatic comparison between hepatoma cell line Hepa1–6 with primary hepatocytes showed that the cell lines were deficient in mitochondria, as a result of re-arrangements occurring in the metabolic pathways. The cell lines also showed high up-regulation of the functions associated with cell cycle and great under-expression of drug metabolizing enzymes, which were typically highly expressed in primary liver cells (Pan *et al.*, 2009). Knowing these changes could be very important in order to predict the *in vivo* situation, on the basis of results obtained using cell lines. It would be interesting to perform a comparative analysis between Caco-2 cells and primary intestine cells, comparing the serotonin transport in the two models, to predict more *in vivo* representative effects for green tea and coffee.

Another limitation of using cells, both cell lines and primary cells, is the extreme simplification of the cell model compared to the *in vivo* situation. When cells are used as part of a tissue or a whole organism, the amount of interactions they have with other structures/organs is enormous, impacting on the molecular processes happening within individual cells.

Moreover, when assessing the effect of compounds on selected cellular functions, it is important to consider that cultured cells are only exposed to the selected compound(s). At the contrary, in the *in vivo* situation, cells are generally exposed to complex matrixes. These matrixes greatly vary, especially in the case of the gastrointestinal tract, for the complexity and diversity of the food and beverages ingested at ones (Spahn-Langguth et al., 1998, Nieto et al., 2013). It would be interesting to design a human study, based on the results obtained

using coffee and green tea on SERT, to compare the effects of these beverages obtained using Caco-2 cells and human volunteers.

6.3. The effect of white tea, green tea and black tea could be assessed on the cells

It is noteworthy that three main teas exist, all produced from *Camellia sinensis*. They are white, green and black tea. The flavour, colour and nutritional properties of each tea are the result of different processes involved to prepare them (Sereshti *et al.*, 2013). The white tea is the least processed and it is not oxidized, thus it is the one with mildest flavour and lower amount of caffeine. It is also the one with the highest polyphenol content (Ji *et al.*, 2002, Tenore *et al.*, 2013). To produce green tea, leaves undergo several steaming or roasting steps (Xu *et al.*, 2012). This results in a reduction of the polyphenol content and in the development of more caffeine (Manning and Roberts, 2003). Finally black tea is a fully fermented beverage. This causes darkening of the leaves, due to tannin development, high caffeine levels and minimal amount of remaining polyphenols, compared to white and green teas (Yang *et al.*, 2000, Di Lorenzo *et al.*, 2013).

It would be interesting to test the effect of white tea and black tea whole extracts respectively on Caco-2 cells and compare the results obtained to those obtained using green tea. This would give useful information on the effect of different caffeine levels on serotonin uptake and on the effect of different food processing methods in terms of serotonin uptake modulation and human health.

6.3. The importance of better investigating dietary food supplements

An interesting area is represented by dietary supplements, which are becoming more and more popular (Umhau *et al.*, 2012). It is very important to investigate and better understand the overall effect of these supplements, in order to ensure concrete health benefits and to limit adverse effects and negative health effects.

A first step in this direction was made in 1994 with the approval of the Dietary Supplement and Health and Education Act (Young and Bass, 1995, Umhau *et al.*, 2012), that stated the need to carry out rigorous studies and trials to assess the beneficial effects and the safe doses of new food ingredients and dietary food supplements. In Europe, the studies on food and supplements and their relationship with human health are regulated from the EFSA (European Food Safety Authority). The main responsibility of the EFSA is to evaluate the scientific validity of health claims coming from food and /or supplement consumption (Birchard, 1999). EU-countries provide the Commission with National lists of health claims. These claims are subsequently evaluated by the EFSA.

The EFSA draws up lists of health beneficial compounds, dividing them in botanical compounds and non-botanical ones. The EFSA also provides a list of all the food compounds for which health claims were rejected. A different list if used for risk reducing compounds and compounds associated with children development. Certain food supplements have dramatically impacted on improving life conditions and are used nowadays routinely administered. An example is folic acid, or vitamin B12, which in many countries is routinely given during pregnancy. Folic acid has helped to reduce the incidence of neural tube defects, such as *spina bifida* (Scholl and Johnson, 2000). It is noteworthy that many drugs which are commonly nowadays would have been classified as supplements in the past. An example is aspirin, which was first discovered and administered after extracting it from the bark of the willow tree (Girstenbrey, 1983). Thus it would not be surprising if

one day the supplements daily taken today would become part of future pharmaceutical plans. In chapter 5, the effect that supplement concentrations of green tea and green tea individual compounds have on SERT in Caco-2 cell monolayers was described. A human study, based on these results would be very interesting, especially considering the controversial results from various studies. It has been shown that green tea supplements vary highly in their content of health-beneficial components. Moreover claims on the label of these supplements often do not correlate with the actual polyphenol content of an equivalent amount of dry green tea leaves. Finally caffeine content is often overlooked and in many cases data are not reported for this compound. A study carried out in America showed that out of 19 analysed green tea supplements, only 7 labels displayed caffeine content, 11 gave EGCG content and only 5 specified the overall polyphenol content (Seeram *et al.*, 2006). In this study caffeine, EGCG and total polyphenol content was measured to vary from 20 to 183 %, from 12 to 143 % and from 14 to 36 %, respectively. The data reported highlight the need for more standardized procedures used to prepare green tea supplements and for more accurate information to be provided on the label. The need to do long term clinical trials to assess the effects of green tea supplements on human health, based on standardized administered doses, also seems evident. In fact, it is likely that despite the predicted health beneficial effects of green tea supplements or daily green tea consumption, their actual interaction with other dietary components would modify the overall outcomes.

There are several considerations to make about human studies designed to test dietary supplements. The introduction of the Dietary Supplement and Health and Education Act has meant the introduction of stricter laws to be applied to even natural ingredients, which in the past could be sold without undergoing long trials (Ashar, 2010). The set of laws approved shorten the distance, in terms of health and safety assessment, between pharmaceutical

drugs and natural products, even though the clinical safety assessment for the pharmaceutical is still more complex and is more time-consuming.

This increase in the time, costs and ethical implications for testing natural ingredients, such as green tea, has meant that many companies have abandoned the idea of carrying out assessment of a range of compounds (Avenell *et al.*, 2005). However, it vital to to invest more in such trials for the impact that, in the longer term, they could have on improving the average health conditions and to reduce the costs of public health care. In fact, it has been shown that malnutrition and lack of certain health-beneficial compounds are often at the base of many degenerative pathologies linked to oxidative stress, such as cancer (Russell, 2007). In chapter 5, it was shown how multiple compounds from coffee significantly down-regulated SERT, especially 5-FQA which was shown to be a very powerful modulator, at concentrations equivalent to these present in a strong cup of coffee. This results could represent a first step to further assess the potency of 5-FQA, selected coffee components and whole coffee extracts *in vivo*. For example a study based on human volunteers could be designed, having volunteers consuming coffee or coffee components in the form of supplements as part of their normal diet.

6.4. Importance of bioinformatic tools

In chapter 3 the high performance search engine Genevestigator was used to collect information on the anatomical distribution and transcriptional modulation of selected transporters of interest. The work was useful to collect comprehensive information on the transporters. The initial aim of the work was to obtain as much information as possible on the modulation of the genes of interest from food compounds. Using Genevestigator highlighted the lack of information available on polyphenols as transporter modulators. In

fact, only two compounds had been tested, curcumin and apple procyanidins. Using Genevestigator provided a large number of information which are useful to create a comprehensive profile on the responsiveness of the genes of interest to a range of conditions. Bioinformatic tools provide a cheap way to carry out preliminary work on selected genes. Filters can be applied in order to refine the results to better match the research interests.

6.5. Functional foods - the importance of better characterizing their impact on human health

Another area of interest is represented by functional food. Similar considerations to those made for dietary food supplements can be made about functional food. Functional food offers food companies growing opportunities. It represents, at the same time, the possibility for consumers to buy quality food that would target specific health end-points and enhance the beneficial effects coming from consumption. Many foods currently sold on the market contain added elements and are reinforced in health-beneficial components. For example, the majority of breakfast cereals are fortified with iron (Whittaker *et al.*, 2001).

An example of the growing interest in functional food is given by the Swiss food company, Nestlé, which in the last decades has invested over £300 million towards research into functional food and beverages (Adriaanse, 2010). For this purpose the company has created two new units. These new units have the purpose of investigating and developing foods and drinks which may help prevent and/or treat chronic diseases, such a Alzheimer's and diabetes. Many studies are currently carried out to better understand how to connect functional ingredients/compounds and foods. The main aim is to determine which food/health promoting compound combinations result in a more appealing product for the consumer to buy (van Kleef *et al.*, 2005, Horska and Sparke, 2007, Vergari *et al.*, 2010).

However, these studies are mainly marketing-oriented and they do not consider a determinant point: the interaction between the functional compound(s) added and the other components already present in the product. Dietary components are always inserted into a very complex matrix, moreover humans often consume multiple food ingredients at once (Frank *et al.*, 2012). Cell-based studies exist that look at the impact of functional food ingredients on human health (Glei *et al.*, 2003). Cell models are a very useful preliminary tool, to predict the effect of individual compounds or simple foods on certain processes. However the cells are not representative of the *in vivo* situation and often result in observations that are not confirmed by studies involving complex food matrixes or human studies (Yin and Chen, 2008). An example is given from studies carried out on carotenoids, which are compounds proven to correlate with a reduced risk of developing certain chronic pathologies. Since carotenoids are not endogenously produced in humans, they have to be introduced through the diet (Michaud *et al.*, 2000, Osganian *et al.*, 2003). *In vitro* studies initially resulted in identification of very high absorption of carotenoids, linked to high presence in the blood stream and therefore to potential for great beneficial health effects (Rauscher *et al.*, 1998). However when comprehensive studies were carried out, considering the interaction between carotenoids and other food compounds, the results showed that the many other food compounds affected their absorption. For example it was shown that fibre significantly reduced the absorption of these compounds (Riedl *et al.*, 1999). This result is extremely interesting, since carotenoids are compounds found in vegetables and thus their consumption is always linked to that of fibre. Taking these considerations into account, it would be interesting to test the green tea and coffee effect on SERT, using a range of food matrices, in a human study. This would help to assess the impact of other ingredients on the overall effect of green tea and coffee on serotonin uptake.

In chapter 5, over 30% of the observed coffee effect on SERT was attributed to specific individual coffee compounds, although the rest of the effect was not explained. It would be

interesting to carry out further studies to characterise the rest of the down-regulatory effect observed on Caco-2 monolayers. There are mainly two possibilities to explain the remaining observable effect on SERT. One is the involvement of other coffee components that have not been tested in this work. Another possibility is the interaction between different compounds: it is possible that the overall observed effect is the result of the different compounds interacting with each other and that the observed result of an individual compound is not necessarily representative of the effect that the same compounds would have if tested in combination with other components.

6.6. The impact of genetic variations on transporter modulation

An important consideration to be made, when evaluating polyphenol absorption, is that transporter expression varies among human beings and it is strictly related to individual genetics (Benet, 2012). Thus, it is important to remember that, when predicting the impact of transporter modulators on human health, the initial genetic of the host plays a determinant role. Each individual represents a unique situation to be considered when designing drugs and therapies.

Awareness of the genetic variation should be the object of more interest when predicting the pharmacological kinetics and the effects of drugs. General and reliable predictions can be made only for biological processes which are based on transporters and enzymes with limited genetic variation. It would be interesting to determine the genetic variation of transporters which are crucial for specific processes, such as serotonin uptake. This would be useful to determine the impact of this variation on pathologies such as depression and eating disorders, linked to poor serotonin presence. This could be done, for example, using tissue samples from a range of donors presenting symptoms of these pathologies and

relating the results of gene quantification in these samples to the analysis of tissues from healthy individuals.

Nowadays the most used therapeutic approach to the cure of pathologies such as depression and eating disorders is based on the use of drugs targeting serotonin and other dopaminergic neurotransmitters. Many of these drugs target re-uptake transporters, such as SERT (Hatta *et al.*, 2013). Though, prescription of these drugs does not take into account the individual variation. Administration of these drugs has often shown to cause problems, such as bone resorption, which may increase the risk of bone fractures (Diem *et al.*, 2007, Shea *et al.*, 2013). It has been hypothesized that the effect of these drugs on bone health is due, at least to an extent, to the presence of a dopaminergic pathway in bone formation (Warden *et al.*, 2005). In fact studies carried out in rats showed that rats expressing a null mutation in the gene encoding for 5-HT had a dysfunctional bone formation (Nandhu *et al.*, 2011).

The general doses of drugs for depression and eating disorders are standardised mainly according to the age and the gender of the patient and do not consider other factors, such as the genetic variation (Rozzini *et al.*, 2008). It could be very useful to introduce a test evaluating the gene levels, as one of the preliminary criteria for patient assessment. This could give more details on the specific situation of an individual and allow adjustment of the pharmacological does. Another important reason to know the genetic variation would be the possibility to introduce natural dietary supplements as an aid to the drug therapy.

For example, in the case of serotonin-dependent eating disorders, the case scenario would be considerably different if the patient displayed abnormally high levels of SERT or normal levels of SERT but abnormally low levels of serotonin synthesis. In the second case the traditional antidepressants, that act by inhibiting SERT and therefore reducing the re-uptake of serotonin, would only represent a very limited part of the therapy. Dietary supplements containing serotonin or serotonin precursors would contribute to restoring normal serotonin levels.

A similar example is given by OAT3. Literature typically describes OAT3 as only significantly expressed in the kidney, as discussed in chapter 3. Studies have identified ten different coding-region variants for *SLC22A8*, resulting in an a slightly different amino acid sequence for OAT3, in DNA samples taken from 270 donors (80 African-Americans, 80 European-Americans, 60 Asian-Americans, and 50 Mexican-Americans) (Erdman *et al.*, 2006). The interesting result of this work is that, among the ten *SLC22A8* forms, only three of them had allele frequency >1 % in a particular ethnic group. However 3.5% of the Asian-American donors expressed an *SLC22A8* form which is associated with reduced ability of OAT3 to transport estrone sulfate. The same form does not affect OAT3 ability to transport cimetidine. Both estrone sulfate and cimetidine are model substrates for OAT3 (Aslamkhan *et al.*, 2006). These findings suggest that OAT3 does not vary a lot among individuals of the same ethnic group and highlights the existence of gene variations between different ethnic groups. Estrone sulfate is an important marker in certain cancers, such as prostate and breast cancer (Purohit et al., 1999, Giton et al., 2008). Increase of estrone sulfate is displayed from patients with these pathologies. It could be interesting to consider that there is only little variability of the gene OAT3, among particular ethnic groups, when designing cancer therapies and predicting anti-cancer pharmacokinetics.

6.7. Conclusions

It is important to investigate the role that our diet has on human transporters, due to the important role that these compounds have in controlling a variety of physiological processes (Srimaroeng *et al.*, 2008, Markadieu and Delpire, 2014). The present work sheds light on some of the aspects of transporter expression and modulation. There are still many grey areas on the topic and much more work will need to be carried out to clarify multiple aspect of transporter modulation, in response to dietary compounds such as polyphenols.

This work summarises the existing knowledge on the anatomical distribution and the modulation of selected transporters. This information could be very useful as a starting point to design comprehensive modulation studies. The work also predicts that the liver HepG2 cell line is an unsuitable model, to assess transporter modulation. In fact the expression patterns of this cell line is very different from human hepatocytes. When investigating the modulation of the organic anion transporter 3, none of the stressors tested showed to have any effect on the transporter. Moreover, uptake of the metabolite kaempferol-3-O-glucuronide was investigated in the cell line: despite an energy-dependent uptake mechanism has been predicted further studies to characterise this mechanism were unsuitable due to the poor uptake rate in the cell model.

Finally the present work showed for the first time that green tea and coffee act as modulators of serotonin re-uptake in the intestine model Caco-2 cells. Both whole extracts and selected individual components acted as concentration-dependent modulators and could be further investigated as potentially useful to treat gut dysfunctions, such as irritable bowel syndrome.

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Appendix I

Anatomical distribution of transporters

Transporter	Tissue	Signal Intensity (IQR)	Standard error	N°of samples
OAT3 (*SLC22A8*)				
	Kidney	22996	1472	100
	Cortex	34987	4189	8
	Medulla	13786	4938	8
	Liver	961	43	75
	Cardiovascular system	1372	23	137
	Small intestine	1503	67	5
	Large intestine	954	103	146
OAT1 (*SLC22A6*)				
	Kidney	70681	4665	100
	Cortex	26778	4964	8
	Medulla	8064	2517	8
	Liver	913	44	75
	Cardiovascular system	792	17	137
	Small intestine	919	52	5
	Large intestine	740	47	146

Appendix I – Anatomical distribution of transporters

Transporter	Tissue	Signal Intensity (IQR)	Standard error	N°of samples
OATP1A2 (*SLCO1A2*)				
	Renal/Urinary system	256	4	111
	Liver	292	40	75
	Cardiovascular system	314	7	137
	Small intestine	252	17	5
	Large intestine	205	5	146
OATP1B1 (*SLCO1B1*)				
	Renal/Urinary system	356	9	111
	Liver	37477	1559	75
	Cardiovascular system	370	4	137
	Small intestine	363	8	5
	Large intestine	281	4	146
OATP1B3 (*SLCO1B3*)				
	Renal/Urinary system	340	5	111
	Liver	45681	2519	75
	Cardiovascular system	258	6	137
	Small intestine	365	8	5
	Large intestine	370	20	146
OATP4C1 (*SLCO4C1*)				
	Kidney	21498	1122	100
	Cortex	3255	458	8
	Medulla	3536	379	8
	Liver	1332	121	75
	Cardiovascular system	186	4	137
	Small intestine	204	4	5
	Large intestine	526	38	146

Appendix I – Anatomical distribution of transporters

Transporter	Tissue	Signal Intensity (IQR)	Standard error	N°of samples
MRP2 (*ABCC2*)				
	Kidney	2715	207	100
	Cortex	1169	77	8
	Medulla	726	60	8
	Liver	13174	792	75
	Cardiovascular system	767	21	137
	Small intestine	763	91	5
	Large intestine	400	24	146
MRP3 (*ABCC3*)				
	Kidney	353	8	100
	Cortex	497	19	8
	Medulla	452	17	8
	Liver	426	19	75
	Arterial system	522	24	10
	Aorta	481	22	4
	Coronary artery	549	33	6
	Heart	327	7	121
	Heart atrium	419	19	17
	Heart ventricle	312	7	104
	Small intestine	455	16	5
	Large intestine	418	8	146
BCRP (*ABCG2*)				
	Kidney	2778	206	100
	Cortex	908	58	8
	Medulla	909	75	8
	Liver	13374	835	75
	Cardiovascular system	1292	47	137
	Small intestine	2109	1017	5
	Large intestine	13620	1101	146

Transporter	Tissue	Signal Intensity (IQR)	Standard error	N°of samples
MCT1 (*SLC16A1*)				
	Kidney	2113	228	100
	Cortex	442	52	8
	Medulla	318	19	8
	Liver	2639	177	75
	Arterial system	644	96	10
	Aorta	841	198	4
	Coronary artery	513	28	6
	Heart	2001	108	121
	Heart atrium	2791	494	17
	Heart ventricle	1872	90	104
	Small intestine	1283	439	5
	Large intestine	3671	277	146
MCT7 (*SLC16A2*)				
	Kidney	2455	85	100
	Cortex	2217	117	8
	Medulla	2005	69	8
	Liver	12024	594	75
	Arterial system	2562	284	10
	Aorta	1714	153	4
	Coronary artery	3128	283	6
	Heart	2174	52	121
	Heart atrium	1838	161	17
	Heart ventricle	2229	53	104
	Small intestine	1602	173	5
	Large intestine	820	28	146
SMCT1 (SLC5A8)				
	Kidney	412	31	100
	Cortex	1163	24	8
	Medulla	1078	19	8
	Liver	1004	37	75
	Arterial system	1348	45	10
	Aorta	1365	35	4
	Coronary artery	1337	77	6
	Heart	867	20	121
	Heart atrium	1033	35	17
	Heart ventricle	839	21	104
	Small intestine	1419	109	5
	Large intestine	986	27	146

Appendix II

Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
OAT1							
	Chemicals and Drugs						
		AM580	-0.18	-1.13	0.034	Vehicle (DMSO/EtOH) IL-4/ GM-CSF treated cells	Tuomisto et al., 2008
		Beta-glycerofosfate (intermediate) study 2	-0.23	-1.17	0.050	Diff. Medium exposed bone marrow stomal cells	Stockwin et al., 2008
		Beta-glycerofosfate (late) study 2	-0.56	-1.47	0.002	Diff. Medium exposed bone marrow stomal cells	Stockwin et al., 2008
		BPDE study 2	0.51	1.44	0.027	Vehicle (DMSO) treated amnion epithelial FL cells	Lu et al., 2009
		Echinomycin/Defero xamine	0.48	1.39	0.007	Astroglia cells stimulated with deferoxamine	Bourdeau et al., 2007
		PMA study 5	0.39	1.31	0.047	ASPC-1 cells	Murphy et al., 2010
		R547 (6h)	0.41	1.34	0.034	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (24h) study 5	0.23	1.17	0.029	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (6h) study 5	0.39	1.32	0.020	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (1h) study 6	0.31	1.23	0.028	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (24h) study 7	0.38	1.29	0.004	Vehicle (DMSO) treated	Fridgway and

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
						DU145 cells	Conlon, 2011
		Sapphyrin PCI-2050	0.32	1.25	0.047	Mannitol treated A549 cells	Wang (Q) et al., 2007
		Sapphyrin PCI-2002 study 2	0.16	1.12	0.031	Mannitol treated A549 cells	Wang (Q) et al., 2007
		Zalypsis study 2	0.61	1.53	0.007	Untreated OPM1 cells	Sarasin-Filipowicz et al., 2008
	Disease						
		Hypertrophic cardiomyopathy	-0.28	-1.22	0.014	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Viral cardiomyopathy	-0.25	-1.20	0.012	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Ischemic cardiomyopathy	-0.19	-1.14	0.008	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Ischemic cardiomyopathy (after VAD)	-0.40	-1.34	0.029	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
	Growth factors and cytokines						
		Il-17	-0.49	-1.41	0.024	Untreated aortic vascular smooth muscle cells	Tallides and Eid, 2008
	Hormones						
		17-betaestradiol (intermediate) study 10	0.37	1.29	0.005	Vehicle treated MCF-7 cells	Johnson et al., 2006
OAT3							
	Chemicals and Drugs						
		BPDE study 2	0.34	1.26	0.016	Vehicle (DMSO) treated amnion epithelial FL cells	Lu et al., 2009
		Cadmium	0.27	1.21	0.014	Untreated, 72h starved NPrEC	Bakshi et al., 2008

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
						cells	
		Doxycycline	0.18	1.13	0.040	Untreated, stably transfected SW480 cells	Gerin et al., 2007
		Ciclosporin study 2	0.23	1.17	0.006	Normal PBMC sample	Grundberg et al., 2008
		Sirolimus	0.23	1.17	0.006	Normal PBMC sample	Brouard et al., 2010
		Calcitriol	0.22	1.16	0.007	Untread breast cancer cells	Murphy, 2010
		Echinomycin/Deferoxamine	0.43	1.35	0.004	Astroglia cells stimulated with deferoxamine	Roth, 2007
		Lypopolysaccharide	0.38	1.30	0.007	Vehicle DTT/ polym.B treated monocytes	Fulcher, 2006
		R547 (4h)-study 4	0.25	1.18	0.044	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (6h)- study 4	0.37	1.29	0.017	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R457 (4h)-study 5	0.46	1.38	0.032	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (4h)-study 6	0.35	1.28	0.001	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		Sapphyrin PCI-2050	0.23	1.17	0.014	Mannitol treated A549 cells	Wang (Q) et al., 2007
	Disease						
		Diabetes study 2	-0.41	-1.34	0.040	Normal regulatory T-cells	Lu et al., 2009
	Growth factors and cytokines						
		G-CSF	0.27	1.20	0.025	Untreated leukocytes	Buzzeo et al., 2007
		IL-22	0.26	1.19	0.003	Untreated neonatal epidermal keratinocytes	Sarasin-Filipowicz et al., 2008
		IL-24	0.18	1.14	0.013	Untreated neonatal epidermal	Sarasin-Filipowicz

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
						keratinocytes	et al., 2008
	Hormones						
		17-beta estradiol study 10	-0.16	-1.12	0.019	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17-beta estradiol study 11	-0.23	-1.17	0.002	Vehicle treated MCF-7 cells	Johnson et al., 2006
OATP1A2							
	Chemicals and Drugs						
		Curcumin (late)	0.16	1.12	0.032	Untreated PMA diff. U937 cells	Mishra et al., 2008
		Paricalcitol (itermediate)	0.20	1.14	0.026	Coronary smooth muscle cells	Stockwin et al., 2009
		Paricalcitol (itermediate) study 2	0.18	1.13	0.042	Coronary smooth muscle cells	Stockwin et al., 2009
		Propiconazole (itermediate)	0.22	1.17	0.024	DMSO treated hepatocytes	Goetz and Dix, 2008
		Triadimefon (high)	0.26	1.20	0.003	DMSO treated hepatocytes	Goetz and Dix, 2008
		Triadimefon (low)	0.14	1.10	0.006	DMSO treated hepatocytes	Goetz and Dix, 2008
		R547 (4 h) study 4	0.22	1.17	0.018	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R568 (intermediate) study 2	0.29	1.22	0.024	Coronary smooth muscle cells	Rosinski, 2009
		Sangivamycin	0.28	1.21	0.042	MCF-7 cells	Rosinski, 2009
	Disease						
		Dilated cardiomyopathy (after VAD)	-0.13	-1.09	0.017	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Hypertrophic	0.30	1.21	0.028	Myocardial sample (left	Schinke et al., 2004

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		cardiomyopathy				ventricle biopsy)	
	Growth factors and cytokines						
		IL-20	-0.20	-1.15	0.027	Untreated neonatal epidermal keratinocytes	Sarasin-Filipowicz et al., 2008
		TGF-β (intermediate) study 5	-0.13	-1.10	0.043	Untreated A549 cells	Shalhoub et al., 2010
		TGF-β3 (intermediate)	-0.14	-1.10	0.026	Untreated mesenchymal stem cells	Mrugala et al., 2009
		TGF-β3 (late)	-0.13	-1.09	0.038	Untreated mesenchymal stem cells	Mrugala et al., 2009
OATP1B1							
	Chemicals and Drugs						
		BPDE (intermediate) study 2	-0.18	-1.13	0.043	Vehicle (DMSO) treated amnion epithelial FL cells	Lu et al., 2009
		BPDE (late) study 2	0.09	1.06	0.029	Vehicle (DMSO) treated amnion epithelial FL cells	Lu et al., 2009
		Curcumin/hydrogen peroxide (early)	0.30	1.23	0.025	Exposed (H2O2)/PMA diff. U937 cells	Mishra et al., 2008
		Actinomycin D	-0.14	-1.10	0.045	Mannitol treated A549 cells	Wang (Q) et al., 2007
		Gefitinib	-0.44	-1.36	0.027	Wild type A431 cells	Guix et al., 2008
		Hydrochloric acid	-0.20	-1.16	0.023	Breast epithelial cells exposed to acidosis	Chen et al., 2010
		Vitamin D3	0.09	1.07	0.009	Vehicle (EtOH) treated bronchial smooth muscle cells	Bossé et al., 2007
		Cycloheximide	0.14	1.10	0.034	Vehicle (DMSO) treated MCF-7 cells	Johnson et al., 2006
		Lipopolysaccharide	0.10	1.07	0.049	Vehicle DTT/ polym.B treated monocytes	Fulcher et al., 2006

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		PMA study 4	0.21	1.16	0.014	ASPC-1 cells	Osaba et al., 2008 (a)
		R547 (6 h)	0.19	1.14	0.017	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (2 h) study 6	0.14	1.10	0.022	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		Sapphyrin PCI-2050	0.41	1.34	0.010	Mannitol treated A549 cells	Wang (Q) et al., 2007
		Sapphyrin PCI-2050 study 2	0.38	1.31	0.017	Mannitol treated A549 cells	Wang (Q) et al., 2007
		Sapphyrin PCI-2002/ZnOAc2	0.09	1.06	0.023	Mannitol treated A549 cells	Wang (Q) et al., 2007
	Disease						
		Viral cardiomyopathy	0.20	1.14	0.005	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Ischemic cardiomyopathy	0.13	1.09	0.005	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
	Growth factors and cytokines						
		Angiopoietin-1	0.16	1.11	0.040	Sparse human umbelical vein cells	Fukuhara et al., 2008
		G-CSF	0.35	1.28	0.006	Untreated leukocytes	Buzzeo et al., 2007
		IL-4/anti CD40	-0.13	-1.09	0.019	Normal resting B-cells	Nickols et al., 2007
OATP1B3							
	Chemicals and Drugs						
		Beta-glycerophosphate (intermediate)	0.29	1.22	0.022	Diff. Medium exposed bone marrow stomal cells	Stockwin et al., 2008
		BPDE (intermediate) study 2	-0.89	-1.85	0.000	Vehicle (DMSO) treated amnion epithelial FL cells	Lu et al., 2009

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		5-aza-2'-deoxycitidine/TSA study 4	0.55	1.47	0.006	Mock treated bronchial epithelial cells	Milani et al., 2011
		Actinomycin D	0.51	1.43	0.001	Mannitol treated A549 cells	Wang (Q) et al., 2007
		Gefitinib	-0.93	-1.91	0.000	Wild type A431 cells	Guix et al., 2008
		Dexamethasome study 4	0.14	1.10	0.004	Vehicle treated HOb514 cells	Guix et al., 2008
		Mifepristone/dexamethasone	0.12	1.09	0.018	A549 cells	Olsen et al., 2009
		Mifepristone	0.26	1.20	0.002	A549 cells	Olsen et al., 2009
		Deferoxamine	1.13	2.20	0.001	Untreated U251 astroglioma cells	Bourdeau et al., 2007
		Tibolone	0.11	1.08	0.038	Untreated endometrium sample	Hanifi-Moghaddam et al., 2007
		Calcitriol (early)	0.18	1.13	0.016	Untread breast cancer cells	Wermuth et al., 2010
		Calcitriol (late) study 2	-0.16	-1.12	0.037	Untread breast cancer cells	Wermuth et al., 2010
		Paricalcitol (late)	-0.13	-1.10	0.037	Coronary smooth muscle cells	Stockwin et al., 2009
		Cycloheximide	0.19	1.14	0.032	Vehicle (DMSO) treated MCF-7 cells	Johnson et al., 2006
		PMA study 2	0.20	1.15	0.005	Mock treated transduced MONO-MAC-6 (shRNA contr.)	Osaba et al., 2008 (b)
		PMA study 4	-6.76	-108.44	0.000	Vehicle (DMSO) tretaed ASPC-1 cells	Murphy, 2010

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		R547 (2 h)	0.50	1.42	0.007	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (24 h) study 2	0.70	1.61	0.012	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (2 h) study 3	0.35	1.28	0.040	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (24 h) study 6	-0.50	-1.43	0.028	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (2 h) study 6	-0.30	-1.23	0.046	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R568 (early)	0.13	1.09	0.046	Coronary smooth muscle cells	Stockwin et al., 2009
		R568 (late) study 2	-0.20	-1.15	0.014	Coronary smooth muscle cells	Stockwin et al., 2009
		Sapphyrin PCI-2050	0.59	1.51	0.006	Mannitol treated A549 cells	Wang (Q) et al., 2007
		Sapphyrin PCI-2050 study 2	0.39	1.31	0.011	Mannitol treated A549 cells	Wang (Q) et al., 2007
		Sapphyrin PCI-2002/ZnOAc2	0.29	1.22	0.034	Mannitol treated A549 cells	Wang (Q) et al., 2007
	Disease						
		ischemic cardiomyopathy (after VAD)	0.12	1.09	0.042	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Unstable angina (30 d)/unstable angina (7 d)	0.15	1.11	0.033	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Barrett's esophagus (acid) (late) study 2	-0.46	-1.38	0.043	CP-A hTERT cells	Donnellan and Hardie, 2007
		Ulcerative colitis	0.92	2.19	0.048	Colonic mucosal endoscopic	Sa et al., 2007

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		study 4 (Inflamm.)				pinch biopsies	
	Growth factors and cytokines						
		G-CSF	0.18	1.13	0.032	Untreated leukocytes	Buzzeo et al., 2007
		SDF study 2	-0.42	-1.34	0.017	Untreated MDA Mb231 cells	Nickols et al., 2007
		SDF study 4	-1.99	-4.17	0.000	Untreated MDA Mb231 cells	Nickols et al., 2007
		TNF-α	-0.24	-1.18	0.024	Normal lymphatic endotelial cells biopsies	Kirshner et al., 2008
	Hormones						
		17beta-estradiol study 8	-0.25	-1.19	0.008	Vehicle treated MCF-7 cells	Johnson et al., 2006
		Estrogen (intermediate)	-0.23	-1.17	0.029	MCF-7 cells	Carroll et al., 2006
		Estrogen (late)	-0.31	-1.24	0.011	MCF-7 cells	Carroll et al., 2006
OATP4C1							
	Chemicals and Drugs						
		5-aza-2'-deoxycitidine/TSA study 5	1.04	2.07	0.004	Mock treated bronchial epithelial cells	Milani et al., 2011
		Gefitinib	-0.22	-1.17	0.041	Wild type A431 cells	Guix et al., 2008
		Peginterferon study 4	0.49	1.48	0.011	PBMC cells	Sartor et al., 2009
		Tibolone	-0.08	-1.06	0.012	Untreated endometrium sample	Hanifi-Moghaddam et al., 2007
		Lipopolysaccharide study 3	-0.23	-1.17	0.015	Untreated THp-1 cells	Fulcher et al., 2006
		PMA study 2	-1.30	-2.47	0.001	Mock treated transduced MONO-MAC-6 (shRNA contr.)	Osaba et al., 2008 (b)
		PMA study 2	-0.71	-1.65	0.033	Mock treated transduced	Osaba et al., 2008

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		(shRNA contr.)				MONO-MAC-6 (shRNA contr.)	(b)
		R547 study 3 (24 h)	-0.23	-1.18	0.021	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 study 6 (24 h)	0.10	1.07	0.030	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		Sapphyrin PCI-5003	-0.13	-1.09	0.046	Mannitol treated A549 cells	Wang (Q) et al., 2007
	Disease						
		Idiopathic dilated cardiomyopathy	0.12	1.09	0.005	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Familial cardiomyopathy	0.15	1.10	0.029	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Postpartum cardiomyopathy	0.18	1.13	0.037	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Viral cardiomyopathy	0.21	1.15	0.002	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Ischemic cardiomyopathy	0.21	1.16	0.001	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Ischemic cardiomyopathy (after VAD)	-0.13	-1.09	0.025	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
	Growth factors and cytokines						
		G-CSF	1.08	2.04	0.003	Untreated leukocytes	Buzzeo et al., 2007
		SDF study 4	-0.43	-1.34	0.001	Untreated MDA Mb231 cells	Nickols et al., 2007
		TGF-β3 (intermediate)/ BMP-2 study 2 (intermediate)	-0.15	-1.11	0.014	Untreated mesenchymal stem cells	Mrugala et al., 2009

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
	Hormones						
		17-betaestradiol (early) study 10	-0.25	-1.19	0.047	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17-betaestradiol (intermediate) study 10	-0.51	-1.42	0.004	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17-betaestradiol (late) study 10	-0.89	-1.85	0.001	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17-betaestradiol study 8	0.20	1.15	0.049	Vehicle treated MCF-7 cells	Johnson et al., 2006
		Estradiol	-0.14	-1.10	0.003	Untreated endometrium sample	Meja et al., 2008
MRP2							
	Chemicals						
		beta-glycerophosphate (short)	0.78	1.75	0.012	Diff. Medium exposed bone marrow stomal cells	Stockwin et al., 2008
		BPDE (intermediate) study 2	-0.59	-1.49	0.010	Vehicle (DMSO) treated amnion epithelial FL cells	Lu et al., 2009
		Rosiglitazone/IL-4/GM-CSF	0.47	1.38	0.021	Vehicle (DMSO/EtOH) IL-4/ GM-CSF treated cells	Tuomisto et al., 2008
		Lactic acid/hypoxia	0.27	1.20	0.013	Untreated breast epithelial cells	Donnellan and Hardie, 2007
		5-aza-2'-deoxycitidine/TSA study 4	0.27	1.21	0.014	Mock treated bronchial epithelial cells	Milani et al., 2011
		Elesclomol/paclitaxel	1.17	2.26	0.015	Vehicle (DMSO) treated Hs 294 T cells	Lu et al., 2009
		Dexamethasone (early) study 3	0.55	1.46	0.011	Vehicle treated HOb514 cells	Guix et al., 2008

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		Dexamethasone (late) study 3	-0.62	-1.54	0.007	Vehicle treated HOb514 cells	Guix et al., 2008
		Dexamethasone study 4	-0.52	-1.43	0.000	Vehicle treated HOb514 cells	Guix et al., 2008
		Mifepristone/dexamethasone study 4	0.46	1.38	0.000	A549 cells	Olsen et al., 2009
		Tibolone	0.13	1.09	0.019	Untreated endometrium sample	Hanifi-Moghaddam et al., 2007
		Echinomycin/deferoxamine	0.92	1.87	0.004	Astroglia cells stimulated with deferoxamine	Bourdeau et al., 2007
		Elesclomol	0.93	1.90	0.003	Vehicle (DMSO) treated Hs 294 T cells	Lu et al., 2009
		Elesclomol study 4	1.64	3.13	0.012	Vehicle (DMSO) treated Hs 294 T cells	Lu et al., 2009
		GW7647 (24 h)	-0.52	-1.44	0.001	Vehicle (DMSO) treated HepG2 cells	Wang (Z) et al., 2007
		GW7647 (6 h)	-0.28	-1.22	0.004	Vehicle (DMSO) treated HepG2 cells	Wang (Z) et al., 2007
		PMA study 2	-0.29	-1.22	0.050	Mock treated transduced MONO-MAC-6 (shRNA contr.)	Osaba et al., 2008 (b)
		PMA study 4	2.13	4.61	0.003	Vehicle (DMSO) tretaed ASPC-1 cells	Murphy, 2010
		R547 (24 h) study 3	0.83	1.77	0.001	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (2 h) study 3	-0.26	-1.20	0.003	Vehicle (DMSO) treated DU145 cells	Murphy, 2010
		R547 (6 h) study 3	0.72	1.67	0.042	Vehicle (DMSO) treated DU145 cells	Murphy, 2010

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		Sapphyrin PCI-5020	-0.55	-1.47	0.001	Mannitol treated A549 cells	Wang (Q) et al., 2007
		Sapphyrin PCI-5020 study 2	-0.30	-1.23	0.034	Mannitol treated A549 cells	Wang (Q) et al., 2007
		Sapphyrin PCI-5002 study 2	-0.32	-1.24	0.009	Mannitol treated A549 cells	Wang (Q) et al., 2007
		UO126 (late)	-0.31	-1.24	0.028	Untreated MCF-7 cells	Roth, 2007
	Disease						
		Ischemic cardiomyopathy	-0.16	-1.12	0.023	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Ulcerative colitis study 4 (Inflamm.)	0.23	1.18	0.002	Colonic mucosal endoscopic pinch biopsies	Sa et al., 2007
		Ulcerative colitis study 4 (Inflamm.)	0.20	1.15	0.018	Colonic mucosal endoscopic pinch biopsies	Sarasin-Filipowicz et al., 2008
	Growth factors and cytokines						
		Angiopoietin-1 (sparse)/angiopoietin-1 (conf)	-0.06	-1.04	0.039	Sparse human umbelical vein cells	Fukuhara et al., 2008
		BMP-2 (early)	0.34	1.26	0.038	Untreated mesenchymal stem cells	Mrugala et al., 2009
		G-CSF	1.08	2.11	0.000	Untreated leukocytes	Bvuzzeo et al., 2007
		IL-20	-0.47	-1.39	0.007		Sarasin-Filipowicz et al., 2008
		KGF	-1.03	-2.05	0.004	Untreated neonatal epidermal keratinocytes	Sarasin-Filipowicz et al., 2008
		TGF-β (intermediate) study 5	-3.17	-8.28	0.002	Untreated A549 cells	Shalhoub et al., 2010
		TGF-β (late) study 5	-3.17	-8.88	0.009	Untreated A549 cells	Shalhoub et al.,

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
							2010
		TGF-β3 (intermediate) study 5/BMP-2 (intermediate) study 2	0.34	1.27	0.005	Untreated mesenchymal stem cells	Mrugala et al., 2009
		TGF-β3 (intermediate)	0.31	1.24	0.047	Untreated mesenchymal stem cells	Mrugala et al., 2009
MRP3							
	Chemicals						
		AG1478 (late)	0.32	1.24	0.028	Untreated MCF-7 cells	Roth, 2007
		beta-glycerophospahe (late) study 2	-0.18	-1.13	0.027	Diff. Medium exposed bone marrow stomal cells	Stockwin, 2008
		curcumin (early)	-0.18	-1.13	0.007	Untreated PMA diff. U937 cells	Mishra et al., 2008
		Rosiglitazone/IL-4/GM-CSF	0.02	1.02	0.025	Vehicle (DMSO/EtOH) IL-4/ GM-CSF treated cells	Tuomisto et al., 2008
		5-azacytidine	-0.17	-1.12	0.029	Mock treaed ovarian cancer cell line	Milani et al., 2011
		Dexamethasone study 4	-0.22	-1.17	0.030	Vehicle treated HOb514 cells	Guix et al., 2008
		Hydrochloric acid	0.12	1.09	0.037	Breast epithelial cells exposed to acidosis	Chen et al., 2010
		Calcitriol (intermediate) study 2	-0.09	-1.07	0.040	Untread breast cancer cells	Wermuth et al., 2010
		Cyclohexemide	0.42	1.34	0.018	Vehicle (DMSO) treated MCF-7 cells	Johnson et al., 2006
		PMA study 3 (shRNA cycT1)	-0.06	-1.04	0.017	Mock treated transduced MONO-MAC-6 (shRNA	Osaba et al., 2008 (b)

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
						contr.)	
		R547 (6 h) study	0.56	1.47	0.001	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (24 h) study 3	0.44	1.38	0.033	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (2 h) study 3	0.17	1.13	0.020	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (6 h) study 3	0.68	1.65	0.046	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (6 h) study 5	0.30	1.23	0.010	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (1 h) study 6	0.14	1.10	0.038	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R568 (intermediate) study 2	-0.19	-1.14	0.002	Coronary smooth muscle cells	Stockwin et al., 2009
		Rottlerin	0.35	1.28	0.047	Normal dermal fibroblasts	Chen and Chi, 2008
		Rottlerin (systemic sclerosis)	-0.51	-1.43	0.024	Normal dermal fibroblasts	Chen and Chi, 2008
		Sapphyrin PCI-5020	0.83	1.77	0.001	Mannitol treated A549 cells	Wang (Q) et al., 2007
		Sapphyrin PCI-5020 study 2	0.79	1.73	0.005	Mannitol treated A549 cells	Wang (Q) et al., 2007
		Sapphyrin PCI-5002	-0.13	-1.10	0.011	Mannitol treated A549 cells	Wang (Q) et al., 2007
		zalypsis study 2	0.29	1.22	0.036	Untreated OPM1 cells	Sarasin-Filipowicz et al., 2008
	Disease						
		Idiopathic dilated cardiomyopathy	0.16	1.11	0.004	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		Viral cardiomyopathy	0.19	1.14	0.031	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
	Grwoth factors and cytokines						
		G-CSF	0.30	1.23	0.018	Untreated leukocytes	Buzzeo et al., 2007
		IFN-g study 2	0.25	1.19	0.016	Untreated OPM1 cells	Sarasin-Filipowicz et al., 2008
		IL-1b	0.22	1.16	0.002	Untreated OPM1 cells	Sarasin-Filipowicz et al., 2008
		IL-22	0.23	1.17	0.013	Untreated neonatal epidermal keratinocytes	Sarasin-Filipowicz et al., 2008
		IL-24	0.20	1.15	0.003	Untreated neonatal epidermal keratinocytes	Sarasin-Filipowicz et al., 2008
		SDF	-0.19	-1.14	0.004	Untreated MDA Mb231 cells	Nickols et al., 2007
		SDF study 2	0.36	1.29	0.033	Untreated MDA Mb231 cells	Nickols et al., 2007
	Hormones						
		17-betaestradiol (late) study 10	-0.24	-1.18	0.021	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17-betaestradiol study 11	-0.05	-1.04	0.021	Vehicle treated MCF-7 cells	Johnson et al., 2006
		estrogen (late)	-0.33	-1.25	0.033	MCF-7 cells	Carroll et al., 2006
BCRP							
	Chemicals						
		Apple procyanidin/TNFa/DMSO	-0.30	-1.23	0.044	HUVEC	García-Conesa et al., 2009
		ARC	-0.53	-1.44	0.025	MCF-7	Szatmari et al., 2006
		BPDE (late) study 2	-0.28	-1.22	0.036	Vehicle (DMSO) treated amnion epithelial FL cells	Lu et al., 2009

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		Rosiglitazone/IL-4/GM-CSF	2.88	6.77	0.009	Vehicle (DMSO/EtOH) IL-4/ GM-CSF treated cells	Tuomisto et al., 2008
		Rosiglitazone/AGN193109/IL-4/GM-CSF	2.63	5.78	0.041	Vehicle (DMSO/EtOH) IL-4/ GM-CSF treated cells	Tuomisto et al., 2008
		Lactic acid	-0.74	-1.67	0.001	Untreated breast epithelial cells	Donnellan and Hardie, 2007
		5-aza-2'-deoxycitidine/TSA study 4	1.82	3.52	0.000	Mock treated bronchial epithelial cells	Milani et al., 2011
		5-aza-2'-deoxycitidine/TSA study 5	1.36	2.56	0.000	Mock treated bronchial epithelial cells	Milani et al., 2011
		Elesclomol/paclitaxel	0.73	1.65	0.049	Vehicle (DMSO) treated Hs 294 T cells	Lin et al., 2007
		Gefitinib	2.25	4.76	0.000	Wild type A431 cells	Guix et al., 2008
		Dexamethasone study 4	-0.29	-1.22	0.000	Vehicle treated HOb514 cells	Guix et al., 2008
		Mifepristone/dexamethasone study 4	0.24	1.18	0.001	A549 cells	Olsen et al., 2009
		Simvastatin (early)	0.26	1.19	0.027	HPBM	van der Meer et al., 2010
		Tibolone	0.76	1.63	0.004	Untreated endometrium sample	Hanifi-Moghaddam et al., 2007
		Epoxomicin (early)	-0.24	-1.18	0.013	HEK293	Chang et al., 2011
		Epoxomicin (late)	-0.85	-1.81	0.000	HEK293	Chang et al., 2011
		Cyclohexemide	0.51	1.43	0.000	Vehicle (DMSO) treated MCF-7 cells	Johnson et al., 2006
		PMA study 2	3.65	13.33	0.005	Mock treated, transduced MONO-MAC-6 cells	Osaba et al., 2008 (b)

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		PMA study 2 (shRNA contr.)	4.50	23.92	0.003	Mock treated, transduced MONO-MAC-6 cells	Osaba et al., 2008 (b)
		PMA study 2 (shRNA cycT1)	3.62	15.84	0.049	Mock treated transduced MONO-MAC-6 (shRNA contr.)	Osaba et al., 2008 (b)
		PMA study 4/PMA study 5	6.22	63.86	0.000	Vehicle (DMSO) tretaed ASPC-1 cells	Murphy, 2010
		R547 (2 h)	0.43	1.34	0.011	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (6 h)	0.85	1.79	0.000	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (6 h) study 2	0.38	1.30	0.022	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (24 h) study 3	-0.59	-1.51	0.002	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (2 h) study 3	0.25	1.19	0.048	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (6 h) study 3	0.69	1.61	0.002	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (24 h) study 5	-0.28	-1.21	0.013	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (24 h) study 5	-0.28	-1.21	0.010	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (24 h) study 6	-0.25	-1.19	0.005	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		Sapphyrin PCI-2002/ZnOAc2	-0.32	-1.25	0.019	Mannitol treated A549 cells	Wang (Q) et al., 2007
	Disease						
		Dilated cardiomyopathy	0.93	1.66	0.006	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		(after VAD) Ischemic cardiomyopathy	0.41	1.26	0.038	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Myocardial infarction (7 d) /unstable angina (7 d)	-0.62	-1.65	0.036	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Ulcerative colitis (Iflamm.) study 4	-2.01	-8.85	0.034	Colonic mucosal endoscopic pinch biopsies	Sa et al., 2007
		Ulcerative colitis (Iflamm.) study 4	-2.74	-8.62	0.004	Colonic mucosal endoscopic pinch biopsies	Sa et al., 2007
	Growth factors and cytokines						
		Angiopoietin-1 (sparse)/angiopoietin-1 (conf)	-3.65	-12.56	0.000	Sparse human umbelical vein cells	Fukuhara et al., 2008
		IL-1b	0.31	1.24	0.029	Untreated neonatal epidermal keratinocytes	Sarasin-Filipowicz et al., 2008
		SDF study 4	-1.09	-2.16	0.007	Untreated MDA Mb231 cells	Nickols et al., 2007
		TGF-β (intermediate) study 5	-3.20	-7.97	0.013	Untreated A549 cells	Shalhoub et al., 2010
		TGF-β (late) study 5	-3.79	-13.17	0.027	Untreated A549 cells	Shalhoub et al., 2010
		TGF-β3 (intermediate)/BPM-2 (intermediate) study 2	-0.12	-1.08	0.022	Untreated mesenchymal stem cells	Mrugala et al., 2009
		TNF-α/Il-1b/IL-6/PGE2	-3.83	-14.01	0.000	Normal lymphatic endotelial cells biopsies	Kirshner et al., 2008
	Hormones						
		17beta-	0.56	1.47	0.000	Vehicle treated MCF-7 cells	Johnson et al.,

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		estradiol/cyclohexemide					2006
		17beta-estradiol (early) study 10	-0.38	-1.30	0.000	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17beta-estradiol (intermediate) study 10	-0.24	-1.18	0.000	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17beta-estradiol (late) study 10	-0.19	-1.14	0.021	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17beta-estradiol study 11	0.31	1.24	0.003	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17beta-estradiol study 12	0.97	1.96	0.009	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17beta-estradiol study 8	1.32	2.53	0.000	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17beta-estradiol study 8	-0.23	-1.17	0.036	Vehicle treated MCF-7 cells	Johnson et al., 2006
		Dihydrotestosterone	-0.50	-1.41	0.023	MCF-7 cells	Carroll et al., 2006
		Estradiol	1.06	1.98	0.000	Untreated endometrium sample	Meja et al., 2008
		Estradiol (late)	-0.31	-1.24	0.001	Untreated endometrium sample	Meja et al., 2008
MCT1							
	Chemicals						
		Apple procyanidin	0.65	1.56	0.001	HUVEC	García-Conesa et al., 2009
		BPDE (early) study 2	0.85	1.80	0.000	Vehicle (DMSO) treated amnion epithelial FL cells	Lu et al., 2009
		BPDE (intermediate) study 2	-0.34	-1.27	0.025	Vehicle (DMSO) treated amnion epithelial FL cells	Lu et al., 2010

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		cadmium (ealry)	-0.82	-1.77	0.005	Untreated, 72h starved NPrEC cells	Bakshi et al., 2008
		Rosiglitazone/IL-4/GM-CSF	-0.41	-1.33	0.029	Vehicle (DMSO/EtOH) IL-4/ GM-CSF treated cells	Tuomisto et al., 2008
		Lactic acid	0.30	1.23	0.013	Untreated breast epithelial cells	Donnellan and Hardie, 2007
		Lactic acid/hypoxia	0.42	1.34	0.023	Untreated breast epithelial cells	Donnellan and Hardie, 2007
		5-aza-2'-deoxycitidine/TSA study 4	0.45	1.36	0.008	Mock treated bronchial epithelial cells	Milani et al., 2011
		5-azacytidine	0.64	1.56	0.024	MEM grown bone marrow-derived mesenchymal cells	Mrugala et al., 2006
		5-azacytidine/tumor cond. medium	0.31	1.24	0.021	MEM grown bone marrow-derived mesenchymal cells	Mrugala et al., 2006
		Dexamethasone study 3 (late)	-0.14	-1.10	0.008	Vehicle treated HOb514 cells	Guix et al., 2008
		Mifepristone/dexamethasone study 4	-0.23	-1.17	0.040	A549 cells	Olsen et al., 2009
		Deferoxamine	0.68	1.60	0.009	Untreated U251 astroglioma cells	Bourdeau et al., 2007
		Peginterferon study 4	-0.30	-1.23	0.004	PBMC cells	Sartor et al., 2009
		Hydrochloric acid	-0.27	-1.20	0.005	Breast epithelial cells exposed to acidosis	Chen et al., 2010
		Tibolone	0.39	1.34	0.022	Untreated endometrium sample	Hanifi-Moghaddam et al., 2007
		Echinomycin/deferoxamine	-1.56	-2.95	0.000	Astroglia cells stimulated with deferoxamine	Bourdeau et al., 2007
		Elesclomol	0.23	1.17	0.017	Vehicle (DMSO) treated Hs	Lin et al., 2007

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
						294 T cells	
		Epoximycin (early)	-0.20	-1.15	0.005	HEK293	Chang et al., 2011
		Epoximycin (late)	0.21	1.16	0.015	HEK293	Chang et al., 2011
		Cyclohexemide	0.17	1.13	0.042	Vehicle (DMSO) treated MCF-7 cells	Johnson et al., 2006
		GW7647 (24 h)	0.18	1.13	0.001	Vehicle (DMSO) treated HepG2 cells	Wang (Z) et al., 2007
		GW7647 (6 h)	0.16	1.12	0.016	Vehicle (DMSO) treated HepG2 cells	Wang (Z) et al., 2007
		PMA study 2 (shRNA cycT1)	-0.49	-1.41	0.002	Mock treated, transduced MONO-MAC-6 cells	Osaba et al., 2008 (b)
		PMA study 3 (shRNA contr.)	-0.96	-1.94	0.020	Vehicle (DMSO) tretaed ASPC-1 cells	Murphy, 2010
		R547 (2 h)	0.30	1.23	0.025	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 study 2 (24 h)	-0.33	-1.27	0.024	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 study 3 (24 h)	-0.67	-1.61	0.003	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 study 3 (2 h)	0.33	1.26	0.015	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 study 4 (6 h)	-0.41	-1.33	0.029	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 study 5 (6 h)	-0.35	-1.27	0.042	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 study 6 (1 h)	-0.38	-1.30	0.045	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 study 6 (4 h)	-0.29	-1.22	0.001	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 study 6 (6 h)	-0.38	-1.30	0.028	Vehicle (DMSO) treated	Mariko, 2006

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
						DU145 cells	
		R568 (intermediate)	-0.20	-1.15	0.033	Coronary smooth muscle cells	
		Rotenone	-0.32	-1.25	0.014		Greene, 2006
		Zalypsis study 2	-1.41	-2.64	0.017	Untreated OPM1 cells	Sarasin-Filipowicz et al., 2008
	Disease						
		Arterial fibrillation study 2	0.55	1.45	0.013	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Ulcerative colitis (Inflamm.) study 4	-1.27	-3.41	0.012	Colonic mucosal endoscopic pinch biopsies	Sa et al., 2007
		Ulcerative colitis (Inflamm.) study 4	-1.93	-4.36	0.000	Colonic mucosal endoscopic pinch biopsies	Sa et al., 2007
	Growth factors and cytokines						
		IFN-a2b	-0.43	-1.36	0.049	Immature dendritic cells	Dhodapkar et al., 2007
		Il-22	0.45	1.37	0.003	Untreated neonatal epidermal keratinocytes	Sarasin-Filipowicz et al., 2008
		IL-24	0.27	1.21	0.027	Untreated neonatal epidermal keratinocytes	Sarasin-Filipowicz et al., 2008
		SDF study 2	0.29	1.22	0.042	Untreated MDA Mb231 cells	Nickols et al., 2007
		SDF study 3 (late)	0.32	1.24	0.010	Untreated MDA Mb231 cells	Nickols et al., 2007
		TGF-β (late) study 5	0.71	1.64	0.047	Untreated A549 cells	Shalhoub et al., 2010
		TGF-β3 (early)/BPM-2 (intermediate) study 2	0.37	1.30	0.048	Untreated mesenchymal stem cells	Mrugala et al., 2009
		TGF-β3 (intermediate)	0.44	1.38	0.048	Untreated mesenchymal stem cells	Mrugala et al., 2009

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		TNF-α/Il-1b/IL-6/PGE2	-0.72	-1.68	0.003	Normal lymphatic endotelial cells biopsies	Kirshner et al., 2008
	Hormones						
		17beta-estradiol (early) study 10	0.31	1.24	0.016	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17beta-estradiol (intermediate) study 10	0.60	1.50	0.046	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17beta-estradiol (late) study 10	0.41	1.33	0.015	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17beta-estradiol study 12	0.97	1.97	0.013	Vehicle treated MCF-7 cells	Johnson et al., 2006
		Dihydrotestosterone	0.08	1.06	0.017	MCF-7 cells	Carroll et al., 2006
		Dihydrotestosterone (early) study 2	-0.20	-1.15	0.016	MCF-7 cells	Carroll et al., 2006
		Dihydrotestosterone (late) study 2	-0.24	-1.18	0.028	MCF-7 cells	Carroll et al., 2006
		Estradiol	0.71	1.65	0.000	Untreated endometrium sample	Meja et al., 2008
		Estradiol/medroxyprogesterone acetate	0.43	1.34	0.004	Endometrium samples	Hanifi-Moghaddam et al., 2007
MCT7							
	Chemicals and Drugs						
		Cadmium (early)	0.51	1.42	0.000	Untreated, 72h starved NPrEC cells	Bakshi et al., 2008
		Lactic acid	0.51	1.43	0.010	Untreated breast epithelial cells	Donnellan and Hardie, 2007
		Lactic acid/hypoxia	0.49	1.40	0.004	Untreated breast epithelial cells	Donnellan and Hardie, 2007

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		5-aza-2'-deoxycitidine/TSA study 4	0.30	1.23	0.004	Mock treated bronchial epithelial cells	Milani et al., 2011
		5-azacytidine	-0.82	-1.77	0.000	MEM grown bone marrow-derived mesenchymal cells	Mrugala et al., 2009
		Gefitinib	-0.70	-1.63	0.000	Wild type A431 cells	Guix et al., 2008
		Calcitriol (intermediate)	0.28	1.23	0.009	Untread breast cancer cells	Wermuth et al., 2010
		Calcitriol (late)	0.15	1.11	0.020	Untread breast cancer cells	Wermuth et al., 2010
		Paricalcitol (intermediate)	0.20	1.16	0.018	Coronary smooth muscle cells	Stockwin et al., 2009
		Vitamin D3	-0.20	-1.15	0.003	Vehicle (EtOH) treated bronchial smooth muscle cells	Bossé et al., 2007
		Echinomycin/deferoxamine	1.20	2.29	0.000	Astroglia cells stimulated with deferoxamine	Bourdeau et al., 2007
		Elesclomol study 2	-0.42	-1.34	0.031	Vehicle (DMSO) treated Hs 294 T cells	Lin et al., 2007
		Epoxomicin (late)	-0.30	-1.23	0.004	HEK293	Chang et al., 2011
		Cyclohexemide	-0.57	-1.48	0.003	Vehicle (DMSO) treated MCF-7 cells	Johnson et al., 2006
		Triadimefon (high)	-0.80	-1.75	0.019	DMSO treated hepatocytes	Goetz and Dix, 2008
		PMA study 4/PMA study 5	-0.60	-1.52	0.019	Vehicle (DMSO) tretaed ASPC-1 cells	Murphy, 2010
		R547 (2 h)	0.33	1.25	0.037	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (6 h)	0.79	1.73	0.000	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 (24 h) study 3	0.66	1.58	0.000	Vehicle (DMSO) treated	Fridgway and

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		R547 (6 h) study 3	0.67	1.59	0.002	DU145 cells Vehicle (DMSO) treated DU145 cells	Conlon, 2011 Fridgway and Conlon, 2011
	Disease						
		Dilated cardiomyopathy (after VAD)	0.41	1.35	0.024	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Idiopathic dilated cardiomyopathy	0.26	1.20	0.000	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Hypertrophic cardiomyopathy	0.35	1.27	0.001	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Viral cardiomyopathy	0.26	1.19	0.005	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Ischemic cardiomyopathy	0.37	1.30	0.000	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
	Growth factors and cytokines						
		BMP-2 (intermediate) study 2	-1.13	-2.10	0.003	HOb514	Muzikar et al., 2009
		BMP-2 (late) study 2	-0.90	-1.85	0.010	HOb514	Muzikar et al., 2009
		G-CSF	0.37	1.30	0.025	Untreated leukocytes	Buzzeo et al., 2007
		IL-1b	0.48	1.39	0.011	Untreated neonatal epidermal keratinocytes	Sarasin-Filipowicz et al., 2008
		IL-20	-0.50	-1.41	0.005	Untreated neonatal epidermal keratinocytes	Sarasin-Filipowicz et al., 2008
		Il-22	-0.47	-1.39	0.003	Untreated neonatal epidermal keratinocytes	Sarasin-Filipowicz et al., 2008
		IL-24	-0.37	-1.29	0.019	Untreated neonatal epidermal	Sarasin-Filipowicz

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
						keratinocytes	et al., 2008
		KGF	-0.98	-1.98	0.001	Untreated neonatal epidermal keratinocytes	Sarasin-Filipowicz et al., 2008
		SDF (late) study 3	0.36	1.30	0.047	Untreated MDA Mb231 cells	Nickols et al., 2007
		SDF study 4	-0.90	-1.84	0.000	Untreated MDA Mb231 cells	Nickols et al., 2007
		TGF-β (late) study 5	3.87	14.63	0.000	Untreated A549 cells	Shalhoub et al., 2010
		TGF-β1 (early)	0.41	1.32	0.033	Untreated A549 cells	Shalhoub et al., 2010
		TGF-β3 (early)/BPM-2 (intermediate) study 2	0.66	1.51	0.044	Untreated mesenchymal stem cells	Mrugala et al., 2009
		TGF-β3 (intermediate)	-0.47	-1.39	0.028	Untreated mesenchymal stem cells	Mrugala et al., 2009
	Hormones						
		17beta-estradiol/cycloheximide	-0.64	-1.55	0.000	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17beta-estradiol (early) study 10	-0.17	-1.12	0.012	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17beta-estradiol (intermediate) study 10	-0.41	-1.33	0.006	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17beta-estradiol (late) study 10	-0.71	-1.63	0.002	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17beta-estradiol study 8	-0.37	-1.30	0.004	Vehicle treated MCF-7 cells	Johnson et al., 2006
		17beta-estradiol study 8	-0.29	-1.22	0.043	Vehicle treated MCF-7 cells	Johnson et al., 2006

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		Dihydrotestosterone	-0.26	-1.20	0.013	MCF-7 cells	Carroll et al., 2006
		Estrogen (early)	-0.28	-1.21	0.015	MCF-7 cells	Carroll et al., 2006
		Estrogen (intermediate)	-0.26	-1.20	0.002	MCF-7 cells	Carroll et al., 2006
		Estrogen (late)	-0.44	-1.35	0.001	MCF-7 cells	Carroll et al., 2006
SMCT1							
	Chemicals and Drugs						
		Apple procyanidin/TNFa/DMSO	0.23	1.18	0.008	Normal lymphatic endotelial cells biopsies	Kirshner et al., 2008
		ARC	-0.33	-1.25	0.000	MCF-7	Szatmari et al., 2006
		beta-glycerophosphate (late) study 2	-0.29	-1.22	0.048	Diff. Medium exposed bone marrow stomal cells	Johnson et al., 2006
		BPDE (intermediate) study 2	0.31	1.24	0.044	Vehicle (DMSO) treated amnion epithelial FL cells	Lu et al., 2009
		5-aza-2'-deoxycitidine/TSA study 4	-0.28	-1.22	0.024	Mock treated bronchial epithelial cells	Milani et al., 2011
		5-azacytidine	-0.18	-1.13	0.006	MEM grown bone marrow-derived mesenchymal cells	Mrugala et al., 2009
		5-azacytidine study 5	0.16	1.12	0.036	MEM grown bone marrow-derived mesenchymal cells	Mrugala et al., 2009
		Actinomycin D	0.65	1.60	0.024	Mannitol treated A549 cells	Wang (Q) et al., 2007
		Rituximab/methylprednisolone	-0.24	-1.17	0.019	synovium samples	Gutierrez-Roelens et al., 2011
		Dexamethasone 9late) study 3	0.20	1.15	0.021	Vehicle treated HOb514 cells	Guix et al., 2008

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		Echinomycin/deferoxamine	0.49	1.42	0.049	Astroglia cells stimulated with deferoxamine	Bourdeau et al., 2007
		Epoxomicin (late)	0.30	1.23	0.006	HEK293	Chang et al., 2011
		PMA	-0.24	-1.18	0.020	Vehicle (DMSO) tretaed ASPC-1 cells	Murphy, 2010
		PMA study 4/Pma study 5	0.24	1.18	0.026	Vehicle (DMSO) tretaed ASPC-1 cells	Murphy, 2010
		PMA (6 h) study 4	0.38	1.30	0.001	Vehicle (DMSO) tretaed ASPC-1 cells	Murphy, 2010
		R547 (24 h)	0.35	1.28	0.025	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		R547 study 6	-0.19	-1.14	0.049	Vehicle (DMSO) treated DU145 cells	Fridgway and Conlon, 2011
		Sangivamycin	-0.43	-1.35	0.012	MCF-7 cells	Rosinski, 2009
		Sapphyrin PCI-2050	0.16	1.12	0.004	Mannitol treated A549 cells	Wang (Q) et al., 2007
	Disease						
		Dilated cardiomyopathy (before VAD)	-0.26	-1.21	0.047	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
		Myocardial infarction (7 d)/unstable angina (7 d)	-0.17	-1.13	0.029	Myocardial sample (left ventricle biopsy)	Schinke et al., 2004
	Growth factors and cytokines						
		BMP-2 (late)	0.24	1.18	0.030	Untreated mesenchymal stem cells	Mrugala et al., 2009
		TNF-α/Il-1b/IL-6/PGE2	0.27	1.21	0.041	Normal lymphatic endotelial cells biopsies	Kirshner et al., 2008
	Hormones						

Appendix II- Transcriptional modulation of transporters

Transporter	Class of modulator	Treatment	Log(2) ratio	Fold change	p value	Sample	Reference
		17beta-estradiol study 11	0.18	1.13	0.031	Vehicle treated MCF-7 cells	Johnson et al., 2006
		Estrogen (late)	-0.18	-1.14	0.046	MCF-7 cells	Carroll et al., 2006

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