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Review

The effects of short-chain fatty acids on the cardiovascular system

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ABSTRACT

The development of cardiovascular diseases is often attributable to loss of endothelial functions of the vascular tissue or to decreased contractile function of the heart muscle. These disturbances are often caused by imbalances in lipid and glucose metabolism. For instance, these imbalances can result in a low-grade inflammatory state of affected endothelial tissue, causing macrophages and fat-rich lipoproteins to accumulate in the subendothelial space. Short-chain fatty acids feature a regulatory function in the cellular metabolism of fatty acids, glucose and cholesterol in various peripheral tissues, both directly as well as at a genetic level. In addition, the strong expression of short-chain fatty acid receptor Ffar2 on various leukocyte populations facilitates a regulatory effect of the fatty acids on various functions of these immune cells. The immunoregulatory effect and influence on lipids, cholesterol and glucose metabolism of short-chain fatty acids can thus contribute to the development of metabolic conditions that promote preservation or recovery of endothelial functions and thereby reduce the risk of development or aggravation of cardiovascular diseases. The current review addresses the effects of short-chain fatty acids on the human cardiovascular system and investigates potential novel interventions for prevention and treatment of cardiovascular disorders using these fatty acids.

Contents

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1. Introduction

Western societies find themselves increasingly confronted with typical nutrition-related diseases, such as obesity and type 2 diabetes mellitus. These diseases often lead to increased risks for the development of several forms of cardiovascular complications [1]. Short-chain fatty acids (SCFAs) are saturated fatty acids featuring an aliphatic chain length of at most eight carbon atoms [2]. The fatty acids are formed by the colonic gut flora from dietary fibres, which manage to escape the host's enzymatic digestive systems in the small intestine. Dietary fibres that manage to reach the large intestine are available for several bacterial fermentative reactions. The fermentation of the different dietary fibres leads to an increase in concentrations of several short-chain fatty acids, especially butyrate, propionate and acetate, in the lumen of the proximal regions of the large intestine [3–5]. The fermentative reactions serve to deliver energy for preservation of the bacterial species and facilitate the excretion of strongly oxidised fatty acids. Thereafter the fatty acids can be absorbed by the enterocytes of the intestines, where they can be further oxidised for the production of energy [6–11].

The beneficial effects of short-chain fatty acids on inflammatory diseases of the intestinal tract and some forms of colon cancer are known for some time. It appears that short-chain fatty acids are able to regulate the inflammatory reactions that contribute to the manifestation of these diseases. They can therefore counteract or even prevent the development of such disorders [12–14]. Short-chain fatty acids can also be absorbed by the enterocytes forming the intestinal wall, whereupon they can be used for the production of energy [9,12,15]. However, a portion of the absorbed fatty acids will not be consumed and can be released via the basolateral membrane to the hepatic portal vein and will reach the systemic circulation after initial liver passage [16,17]. Lesser known are the beneficial effects short-chain fatty acids achieve in the prevention and treatment of various cardiovascular diseases after being absorbed in the circulation [18–20]. In spite of a strong concentration reduction of the fatty acids due to the consumption by enterocytes combined with the ability of the liver to clear large fractions of these acids, even small blood concentrations appear to induce positive effects [21,22]. The *Food and Drug Administration* (FDA) recently acknowledged the claims regarding the use of short-chain fatty acids in the prevention of cardiovascular disease [23,24].

Cardiovascular events are often the result of loss of endothelial function of the blood vessels or reduced contractivity of the cardiac muscle. Disturbance of both functions can often be ascribed to disruptions in lipid and glucose metabolism and generally appear in obese patients or patients who suffer from diabetes [25–27]. After reaching the systemic circulation, short-chain fatty acids prove to be able to regulate the metabolism of various peripheral tissues both directly and on a genetic level. Additionally, butyrate and propionate appear to have anti-inflammatory effects on several immune cells, that could be involved in the development of, for example, atherosclerosis [28–30]. This review illustrates the effects of short-chain fatty acids on the human cardiovascular system and subsequently gives an overview of intervention options in the prevention and treatment of cardiovascular diseases.

2. Synthesis of short-chain fatty acids

In people of Western societies, approximately 20–60 g of the daily intake of carbohydrates evades the digestive and absorptive system of the small intestine and manages to reach the colon [3]. The large intestine houses a large and complex bacterial ecosystem containing around 10^{10} up to 10^{11} cfu per gram wet weight. Having wet weights that vary typically between 250 and 750 g, this implies a total bacterial population close to 10^{13} cfu of the final section of the gastrointestinal tract [31]. The population comprises at least 400 different bacterial species, of which about 99% is anaerobic [3,31,32].

Nutrients that manage to evade the digestive system of the small intestine and enter the large intestine are potential substrates for the bacterial metabolism in the latter organ. The anaerobic bacteria form short-chain fatty acids by degrading these substrates by means of anaerobic respiration and fermentative reactions [3–5]. This leads primarily to the production of fatty acids butyrate, propionate and acetate. In addition some gasses are generated, such as methane, carbon dioxide and hydrogen [6,33,34]. These reactions are executed by bacteria to generate energy and release carbon to warrant microbial growth and preservation of the bacterial specie. However, a fraction of the end products can also be deployed by the host [6–11]. Especially mammals appear to be able to absorb the synthesised short-chain fatty acids from the colonal lumen and generate energy by oxidation of these substrates. Particularly in ruminants the absorption and oxidation of short-chain fatty acids provide a significant portion of their total energy production [6,9]. In Western societies the contribution of short-chain fatty acids to the total energy requirement turns out to be much lower. People that are subjected to a Western pattern diet obtain typically 5–10% of their total energy need by the oxidation of these fatty acids [35].

In spite of the limited contribution of short-chain fatty acids to the total energy requirements of humans, the main purpose of the oxidation of short-chain fatty acids by the colonal mucosa is the production of energy. Herewith, there appears to be a preference for the oxidation of butyrate, followed by propionate and in a lesser extent for acetate [36]. Colonocytes even seem to prefer the degradation of butyrate to that of glucose, as it has essential features for the preservation of the colonocytes themselves [12]. As a matter of fact, the metabolism of butyrate by the colonocytes yields sufficient energy to prevent autophagocytosis; a mechanism involving cell degeneration in order to meet energy requirements [15]. However, a fraction of the short-chain fatty acids is not used for energy production in the colonocytes. This portion of the fatty acids is transported over the intestinal epithelium towards the hepatic portal vein [16,17]. The mechanisms of intracellular transport towards the systemic circulation will be addressed in upcoming chapters.

In 1960 it was thought that microbial production of short-chain fatty acids in the large intestine could cause diarrhea. The general idea was that short-chain fatty acids were poorly absorbed by the epithelial cells of the large, thereby increasing the osmolality of the luminal side of the colon. As result, more water would be retained in the lumen, thus causing watery stool. Currently it is known that approximately 90% of the short-chain fatty acids is absorbed by the intestinal epithelium and there is no existence of an osmotic gradient towards the lumen [37,38]. Contrarily, short-chain fatty acids seem to be able to stimulate the uptake of water and sodium salts from the lumen of the large intestine, resulting in further reduction of the concentration of osmotically active compounds. Therefore it is very unlikely that short-chain fatty acids are able to induce diarrhea [11,38].

2.1. Substrates

Microbial growth and preservation of the bacterial population in the large intestine strongly depend on the supply of suitable substrates for fermentation and anaerobic respiration. As indicated before, substrates need to pass the small intestine that normally decomposes and absorbs a large fraction of these nutrients. Nutrients that are not degraded by the local digestive system are able to reach the more distal areas of the intestine and can be divided into two different classes [38–40]. The first class comprises of *non-digestible* nutrients that can not be degraded as the digestive system lacks endogenous enzymes needed for the decomposition of these substances [41]. The second class of substrates consists of *resistant* nutrients which manage to escape the digestive system in the host's small intestine [42]. Both nutrient classes are available for the bacterial metabolism in the large intestine [17].

Short-chain fatty acids are mainly produced from poly-, oligo- and fructooligosaccharides, proteins, peptides and glycoprotein precursors by anaerobic micro-organisms [3,43]. The fermentation of carbohydrates turns out to be most important mechanism for the production of short-chain fatty acids, as the major part of the bacteria in the large intestine is saccharolytical. Therefore the bacterial population in the large intestine has a large decomposing capability for this type of substrate [17,40]. As a result of the vast variety of micro-organisms in the colon, a strong competition for these complex, non-digestible carbohydrates exists [44,45]. This is confirmed by analyses of the genome of several commensal gut bacteria the large intestine houses. Some bacterial species, such as *Bifidobacterium longum* and *Bacteroides thetaiotaomicron* which feature a genome consisting of 2.26 and 6.26 megabases respectively, dedicate at least 8% of their genomes to the transport and the metabolism of complex carbohydrates [46,47]. When comparing these species with *Escherichia coli*, which has a genome comprising of 4.64 megabases, *B. longum* and *B. thetaiotaomicron* prove to be able to synthesize respectively two times and nine times as much enzymes for the breakdown of carbohydrates [47].

Another factor that can contribute to the fermentation of primarily, non-degraded carbohydrates is the rich supply of the polysaccharide fraction of plant cell wall material. This fraction can not be decomposed by the host's digestive system, which enables large amounts of this substrate to reach the colon out of the ileum [3,11,48].

2.1.1. Polysaccharides from plant cell wall material

Plant cell wall material consists of various polysaccharides such as cellulose and its derivates, lignins, pectins, arabinogalactans and several types of gums and mucilages [3,40]. These non-digestible classes of carbohydrates are designated as *dietary fibres* and can be decomposed by large variety of microbial hydrolases, esterases and lyases [49].

Some constituents of dietary fibres, especially cellulose and lignin, can not or only to a limited extent be broken down by micro-organisms in the large intestine. This incomplete decomposition results in the formation of fragments of vegetable fibres which are transported past the colon towards the more distal regions of the intestine [50]. Only limited research has been conducted to the few anaerobic micro-organisms in the human colon that are capable of fully metabolising cellulose. One of the investigated cellulolytic micro-organisms is *Ruminococcus flaveciens*, a specie within the anaerobic bacterial class *Clostridia*. This specie features a cellulosome, a multi-enzyme complex, at its cell surface providing adhesion and degradation of hard to metabolise cellulose [51]. The cellulosome contains *dockerin*, a protein domain, enabling, via interaction, the fixation of the enzyme complex to *cohesins* in the bacterial cell wall. The multi-enzyme complex also contains some substrate-binding protein domains that facilitate the adhesion to cellulose molecules [50]. This was illustrated by showing a strong adherence between cellulose and the bacteria after isolating the specie from human faecal samples [52,53].

Contrary to cellulose and lignin, the intestinal flora is capable of fully decomposing hemicellulose and pectins. After degradation of the primary cell walls of the dietary fibres by other bacteria in the large intestine, the end products are able to freely dissolve from the amorphous matrix of the cell wall into the intestinal fluid and become available as substrates for further metabolization by other bacteria in the large intestine [50].

One of the classes of substances that is released from the cell wall matrix consists of xylans [3,50]. These heterogeneous polymers, that belong to the hemicelluloses, comprise of β-1,4-

xylose, acetyl, arabinosyl and glucuronic acid residues. Only a few bacterial species turn out to be capable of degrading xylans [50]. In a study on *Bacteroides ovatus*, one of the previously mentioned bacterial species, an operon regulating the synthesis of xylan-degrading enzymes could be identified [54]. Other bacterial species in humans that are also capable of degrading xylans include *Roseburia intestinalis* and *Ruminococcus flaveciens* [55].

Another class of substances that is released during the decomposition of the primary cell wall consists of pectins [3,50]. Pectins comprise of long, linear chains of α-1,4-glucuronic acid residues, possibly featuring partial esterification with methanol or branching with rhamnogalacturonans. In the latter case, the side chains of the branches consist of several saccharides, including D-xylose, L-fucose and D-glucuronic acid. From investigation on the speed of fermentation it was found to be dependent on the level of methylation and the degree of branching of the pectins [50,56]. Research of human faecal samples showed that full decomposition of pectins can be achieved within 24 h by the micro-organisms present in the faeces [56].

2.1.2. Resistant starches

Not all starches can be fully digested by the host [3,11,40]. Some types appear to be insensitive to the gelatinisation that occurs during the boiling of starches and therefore remain non-digestible. Such starches can not be broken down by the host's amylases and consequently can not be absorbed in the small intestine. Hence this fraction, which represents about 10–15% of the starch intake, is capable of reaching the large intestine [11,57]. This non-digestive portion is known as *dietary starch* or *resistant starch* [6].

Dietary starch consists of amylose, which comprises of α-1,4-glucose residues and amylopectin, a branched polymer of amylose, that is connected to the amylose backbone by bonds at the α-1,6 positions. Research has shown that the commensal gut flora is better capable of degrading starches that contain relatively large amounts of amylopectin in comparison to starches with low amounts [50,57].

The intestinal flora deploys three different classes of hydrolases to decompose starches. The first class of starch-hydrolysing enzymes is represented by *α-amylases*. These enzymes are capable to break the α-1,4 bonds between the glucose residues and the amylose backbone. The second class concerns *type I pullulanases*, which are able to separate the amylopectin branches from the amylose backbone by breaking the bonds at the α-1,6 positions. The third class of hydrolases includes *amylopullulanases*, which are capable of breaking both bonds at the α-1,4 as well as at the α-1,6 positions [57]. This was demonstrated by an investigation where testees administered a human α-amylase inhibitor, whereafter increased faecal butyrate concentrations where found [58]. Butyrate is one of the major end products of bacterial fermentative reactions in the large intestine [6,34,59]. This demonstrated that inhibition of starch-degrading enzymes in the small intestine provided a larger availability of starch in the large intestine for bacterial fermentation [58].

Investigation of the starch-degrading system of *Bacteroides thetaiotaomicron*, a gram-negative bacterial specie, has shown that its genome contains multiple genes that code for enzymes that are involved in adhesion, hydrolyses and transport of created fragments from the extracellular environment to the internal environment of the bacteria [47]. First, adhesion of *B. thetaiotaomicron* to the dietary starch occurs. This attachments provides optimal positioning of the substrate, which lowers the required activation energy for hydrolysis, thus attempting to optimize the hydrolytical activity [60]. After the adhesion hydrolysis of the bound starch takes place involving membrane-bound protein SusG [61]. Thereafter starch fragments are transferred to the periplasm, the region between the inner and outer membrane present in gram-negative bacteria, where complete hydrolysis of these fragments occurs [50].

Certain gram-positive bacteria featuring a high guanine-cytosine fraction in their genome, as present in several *bifido* species, seem to have amylolytical properties and possess a designated cell surface to adhere to starch granules. Research has shown that although substrate adhesion is not essential for these bacterial species in order to degrade starches, the presence of this specific adhesion surface is characteristic for these bacteria [62].

Gram-positive bacteria containing small guanine-cytosine fractions in their genome can also contribute to the degradation of starches. For example, *Butyrivibrio firbisolvens* and *Roseburia inulinivorans A2-194* can produce large α-amylase enzymes consisting of 1333 and 1674 amino acids respectively [63]. The synthesised proteins comprise of several domains, including α-amylase catalytic domains belonging to family 13 of the glycoside hydrolases [64]. It is also assumed that these enzymes contain several carbohydrate binding domains and a designated carboxyl tail domain that facilitates adhesion to the bacterial cell wall. The positioning of the enzyme by the carboxyl tail domain of the protein possibly has a beneficial effect on the hydrolysis activity by lowering the required activation energy [63].

2.1.3. Inulin and fructooligosaccharides

Both inulin and fructooligosaccharides are linear polymers of fructose monomers combined with a terminal glucose residue, which are connected by bonds at β-2,1 positions. Inulin and fructooligosaccharides differ in the level of polymerisation. The degree of polymerisation of inulin typically ranges from 3 to 60 monomeric units, whereas fructooligosaccharides features 3–9 monomeric units [3,50]. Research involving fluorescent *in situ* hybridization to investigate the feasibility of administering these substrates in order to selectively induce the growth of butyrate-producing bacteria species revealed that these substrates can especially stimulate bifidobacterium, lactobacillus and *Roseburia inulinivorans*. Following from this, it can be deducted that these substrates may be applied in prebiotic formulations [65,66].

Degradation of inulin and fructooligosaccharides involves several bacterial β-fructofuranosidases, which differ in the level of activity in breaking bonds at the β-2,1 positions in sucrose, inulin and fructooligosaccharides [67]. It appears that β-fructofuranosidases that are synthesised by *Bifido breve* are the most active agents in breaking bonds between the fructose monomeric units and the terminal glucose residue [68], whilst *Bifido lactis* produces a variant of β-fructofuranosidase that is especially effective in breaking bonds between fructose monomeric units [69]. However, both variants of the enzyme appear to be far less effective in breaking bonds in longer inulin molecules [68,69].

Other research involving 11 species of the *bifidobacterium* genus were independently administered inulin or fructooligosaccharides, proved that only 5 of the tested species where actually capable of degrading inulin. Nevertheless, all investigated *bifido* species were able to decompose fructooligosaccharides. However, all species were able to survive on administered inulin when the species were combined. The investigators attributed this phenomenon to the so-called *bifidogenic effect* where species that are able to decompose inulin release monosaccharides after fermentation that can be used by the other species as a nutrition source. Through this there appears to exist a form of commensalism between the different *bifido* species, potentially featuring a form cross-feeding [70].

2.1.4. Simple sugars and sugar alcohols

These sugars and its derivates are usually rapidly absorbed by the host in the small intestine. As a result these substances offer only a limited contribution as substrates for microbial decomposition in the large intestine. However, in case of lactose intolerance

due to lactase deficiency, the contribution will increase correspondingly. In research where test subjects administered L-lactulose and L-rhamnose, an increase in serum propionate concentration was observed. Neither lactulose or rhamnose can be broken down by the host's digestive system in the small intestine, hence this research indicates that simple sugars and sugar alcohols are able to act as fermentation substrates for the microbiota in the large intestine [50,71].

2.1.5. Endogenous substrates

Apart from the substrates that reach the large intestine which originate from the host's food intake, the body also provides fermentation substrates to the colonic gut flora. Mucous covering the apical membrane of the intestinal wall is the primary endogenous substrate. Released mucous consists of glycoproteins with a composition that approximates 80% carbohydrates and 20% mucins. Several bacterial peptidases and proteases are able to break down mucins to their constituent amino acids, which can be fermented afterwards [72]. Fermentation predominantly results in branched end products, such as isobutyrate, 2-methylbutyrate and isovalerate. These products originate mainly from the fermentation of valine, leucine and isoleucine. However, the contribution of protein substrates to the production of short-chain fatty acids is limited. Therefore the production of these fatty acids strongly depends on fermentation of carbohydrates [73].

2.2. Fermentation

At the beginning of this phase of decomposition by the microbial gut flora in the large intestine, non-digestible carbohydrates have been converted to their elementary, constituent sugars; the monosaccharides [43]. Given the anaerobic environment of the large intestine, present bacteria are not able to use oxygen as oxidizer in the synthesis of highly energetic ATP, which is essential for growth and preservation of bacterial species. This implies that aerobic respiration is not possible in this environment and the present bacteria need to utilize anaerobic respiration and fermentation to release energy from monosaccharides [40]. It has to be noted that the fermentative reactions are responsible for the formation of short-chain fatty acids [6,11]. Anaerobic respiration is mainly involved in oxidizing exogenously produced hydrogen gas by non-organic anions and has a significant role in the regulation of the fermentative reactions [40,74].

In the fermentation of monosaccharides in anaerobic environments the constituent sugars are decomposed and oxidised by means of endogenous electron acceptors. These electron acceptors are often intermediate products of the applicable fermentative reactions, for instance pyruvate or its derivates [3,40,75]. To convert ADP into ATP bacteria deploy *substrate level phosphorylation*, which implies the transfer of a phosphate group from the substrate and consequently results in its reduction. In fact, the phosphate groups needed for ADP phosphorylation are provided by highly energetic intermediate products of the various fermentative reactions [75].

2.2.1. Glycolytic pathways

Monosaccharides formed by microbial degradation in the large intestine can be divided into two types. The first type consists of elementary, constituent sugar molecules featuring six carbon atoms, also known as hexoses. The second type comprises monosaccharides of five carbon atoms, which are known as pentoses. Various glycolytic pathways are involved in the degradation of these types of monosaccharides to phosphoenolpyruvate [75,76]. Currently, three different glycolytic pathways have been identified:

1. Embden–Meyerhof pathway
2. Pentose phosphate pathway
3. Entner–Doudoroff pathway

The Embden–Meyerhof pathway is used for the decomposition of hexoses. This type of glycolytic activity occurs in the bacterial cytoplasm and applicable substrates feature a six carbon atom phase and a three carbon atom phase. Hexoses are initially subjected to double phosphorylation in order to form fructose 1,6-biphosphate. Hereafter, this monosaccharide is split into two glyceraldehyde 3-phosphate molecules, which marks the transition from the six carbon atom phase into the three carbon atom phase. Then each of the glyceraldehyde 3-phosphate molecules is oxidised by glyceraldehyde 3-phosphate dehydrogenase to form 1,3-bisphosphoglycerate, at the same time reducing NAD^+ to NADH. Next, the 1,3-bisphosphoglycerate is subsequently converted to phosphoenolpyruvate and then to pyruvate. Per molecule this results in the release of two phosphate groups, which are used for the substrate level phosphorylation of ADP to ATP [75]. A survey of the Embden–Meyerhof pathway is given in Fig. 1.

Pentoses are broken down by means of the pentose phosphate pathway, which also occurs in the bacterial cytoplasm. The monosaccharides featuring five carbon atoms are transformed into various sugar phosphates comprising a number of carbon atoms that ranges between three and seven. These variants of sugar phosphates are formed by transketolase and transaldolase, that respectively remove two and three carbon atoms from the substrate. After undergoing several reactions which are catalysed by these enzymes, glyceraldehyde 3-phosphate is formed from the several sugar phosphates. As glyceraldehyde 3-phosphate is an intermediate product of the Embden–Meyerhof pathway, this compound can be converted into pyruvate using the latter pathway. An alternative option is the subsequent conversion of glyceraldehyde 3-phosphate to fructose 6-phosphate followed by glucose 6-phosphate. Thereafter, the latter sugar molecule can be oxidised to ribulose 5-phosphate, thereby reducing two $NADP^+$ molecules to NADPH. Ribulose 5-phosphate can then re-enter the pentose phosphate pathway [75]. A summary of the pentose phosphate pathway is given in Fig. 2.

Some noteworthy intermediate products in this pathway are erythrose 4-phosphate, which is utilized in the synthesis of aromatic amino acids, and ribose 5-phosphate, that is used in the production of nucleic acids [75].

The pentose phosphate pathway offers bacteria the possibility to form hexoses from pentoses. Hexoses are essential for producing peptidoglycan; an essential constituent of bacterial cell walls. Hence, this pathway enables bacteria to synthesize their cell walls when they are dependent on nutrient sources offering only 5-carbon monosaccharides [75].

The Etner–Doudoroff pathway is not applicable for the microbiota in the large intestine and will therefore not be addressed in this review [40].

2.2.2. Fermentative reactions

As has been indicated in the previous paragraph, the large intestine holds no oxygen that can act as an electron acceptor. Nevertheless, NADH that has been produced by means of the previously described pathways needs to be reoxidised to NAD^+ to prevent stagnation of glycolysis and the oxidation of glyceraldehyde 3-phosphate. This process is facilitated by pyruvate and its derivates, intermediate products of fermentative reactions, that act as electron acceptors. These substances are only partially oxidised, resulting in strongly reduced short-chain fatty acids and gasses as the major end products [75]. This reaction type results in relatively low ATP yields, wherein ATP is formed by substrate level

phosphorylation. Consequently, this implies that for the release of sufficient energy for growth and preservation of involved gut flora large amounts of substrate are required. Next a survey will be given of the three fermentative pathways involved in clearing excess electrons and restoring the redox balance [40,75].

2.2.2.1. Classical fermentation pathway. In the classical pathway, pyruvate is used as an acceptor for the electron released during the oxidation of NADH to NAD^+ and is reduced to lactate or ethanol [78].

2.2.2.2. Formation of hydrogen gas. The partial pressure of microbially formed hydrogen in the lumen of the large intestine has a regulative effect on numerous fermentative reactions [40]. Two different fermentative processes can lead to the generation of hydrogen gas [79].

The first process is exergonic ($\Delta G < 0$). Pyruvate is oxidised to acetyl-CoA and the released electrons are transferred to hydrogen ions, resulting in the formation of hydrogen gas. The two enzymes that are involved in this reaction are pyruvate:ferredoxin oxidoreductase and ferredoxin dehydrogenase [79].

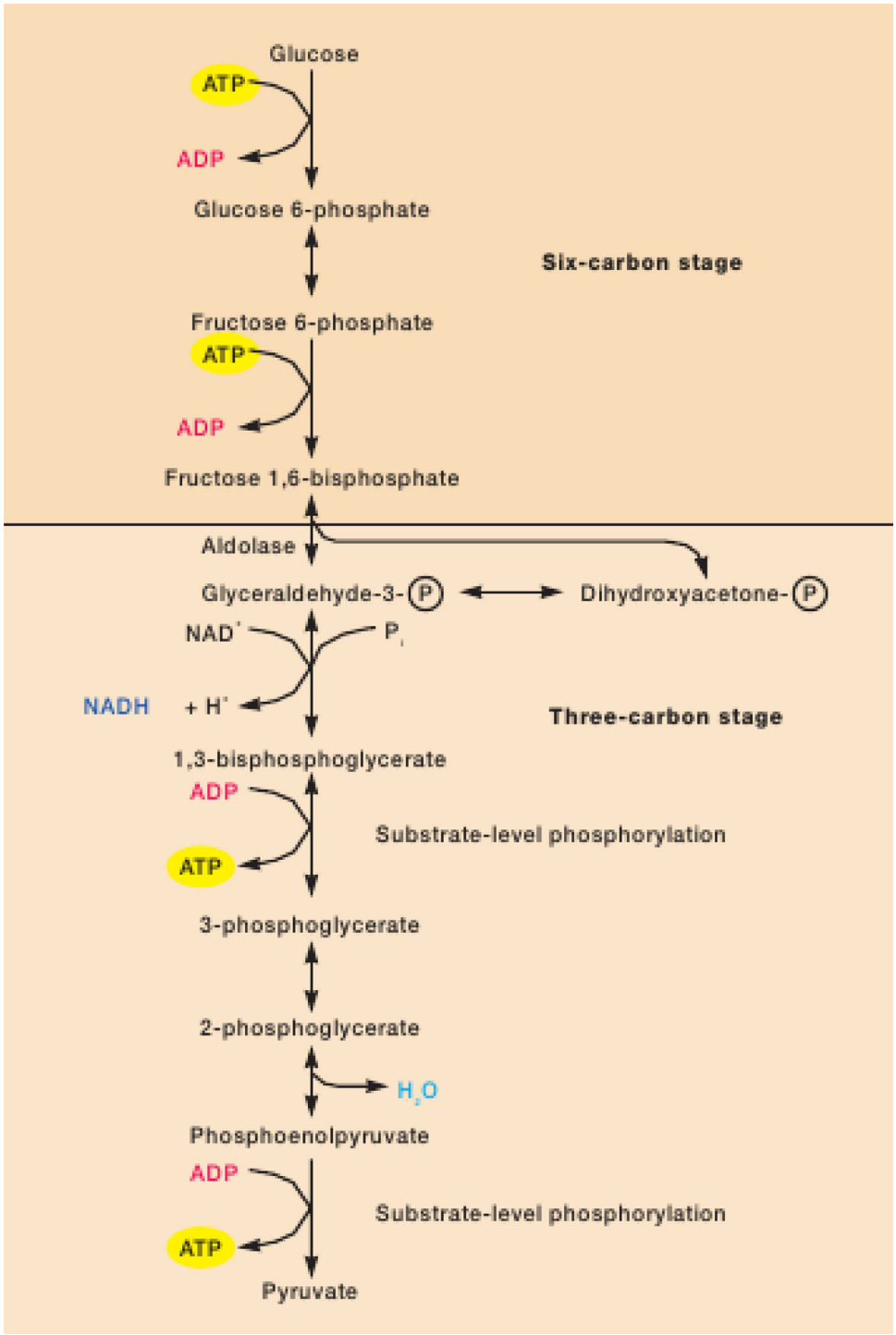

Fig. 1. Embden–Meyerhof pathway [77].

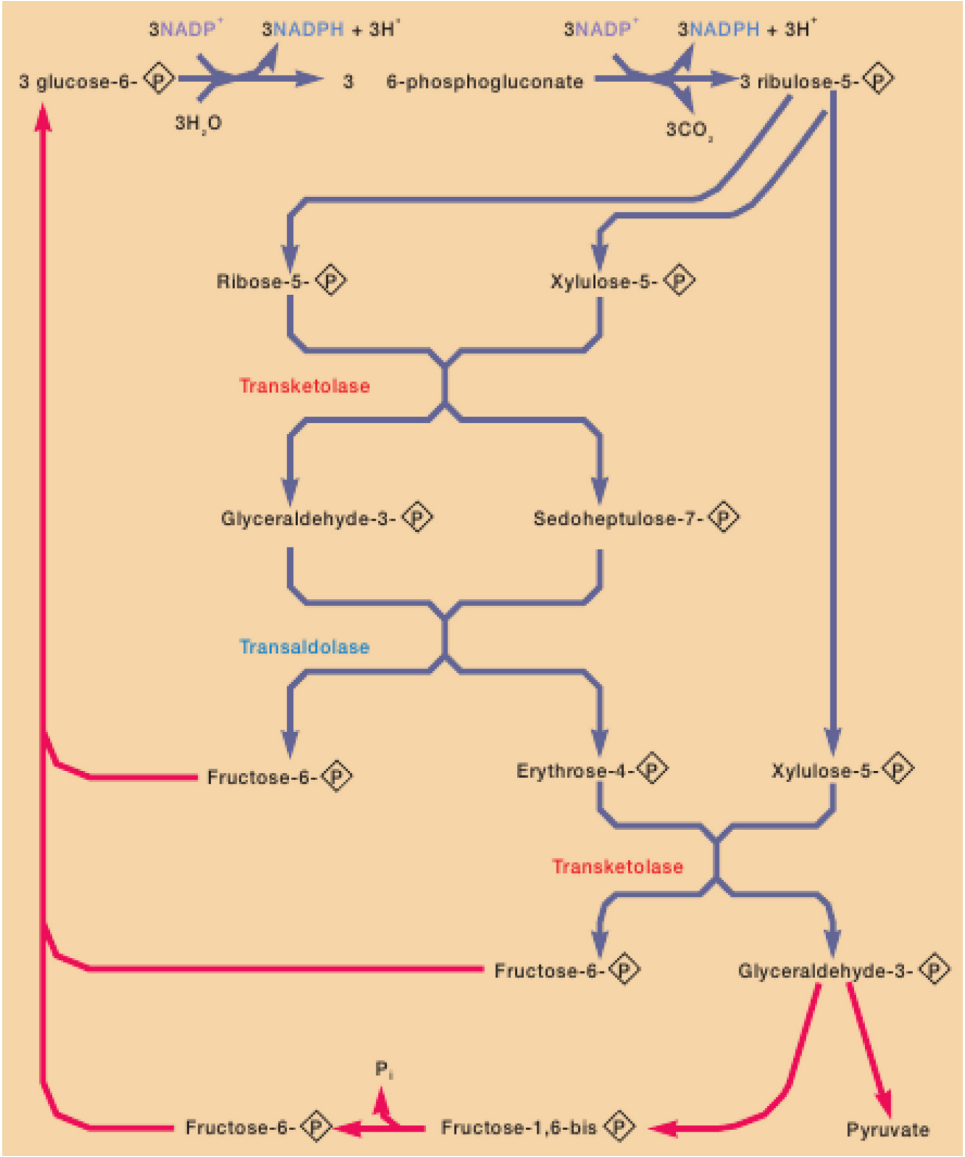

Fig. 2. Pentose phosphate pathway [77].

Contrary to the first process, the second process is of endergonic nature ($\Delta G > 0$). In this reaction NADH is reoxidised to NAD^+ and the released electrons are also transferred to hydrogen ions in order to form hydrogen gas. However, this reaction requires a low H_2 partial pressure in the lumen of the large intestine and is therefore driven by hydrogen consuming bacteria [79].

2.2.2.3. Primitive anaerobic electron transport chain. The reduction of NADH involves the use of a primitive anaerobic electron transport chain. First, phosphoenolpyruvate is carboxylated to oxaloacetate followed by the reduction of the generated oxaloacetate to fumarate. In this process NADH is reoxidised to NAD^+. Fumarate is able to absorb the electrons that are released in the enzymatic oxidation of NADH by NADH dehydrogenase and fumarate reductase, thereby reducing itself to succinate. The remnant protons from this reaction are transported outside the cell membrane by NADH dehydrogenase, thereby developing a chemiosmotic gradient that drives the phopshorylation of ADP to ATP [80,81].

At low carbon dioxide partial pressures in the lumen of the intestines, succinate can be converted into methylmalonate, which is subsequently split into propionate and carbon dioxide. The generated carbon dioxide can then be reused for the carboxylation of phosphoenolpyruvate to oxaloacetate [80,81].

2.2.3. Major fermentation products

Acetate, propionate and butyrate are the main end products of fermentative reactions [73]. Even though other short-chain fatty acids are produced, they are generally involved in *cross-feeding* that eventually results in the conversion of these fatty acids into one of the three earlier mentioned main end products [73,82]. Fig. 3 gives an overview of the major fermentative reactions involved in the formation of these short-chain fatty acids.

2.2.3.1. Acetate formation. Two different pathways exist for the formation of acetate. First of all, acetate can be generated by decarboxylation of pyruvate to acetyl-CoA, which is then converted to acetate by means of hydrolysis [6]. Secondly, the *Wood-Ljungdahl pathway* can also be utilized for the production of acetate. In the latter pathway carbon dioxide is reduced to carbon monoxide, after which the resulting carbon monoxide reacts with both a methyl group and a coenzyme A molecule to form

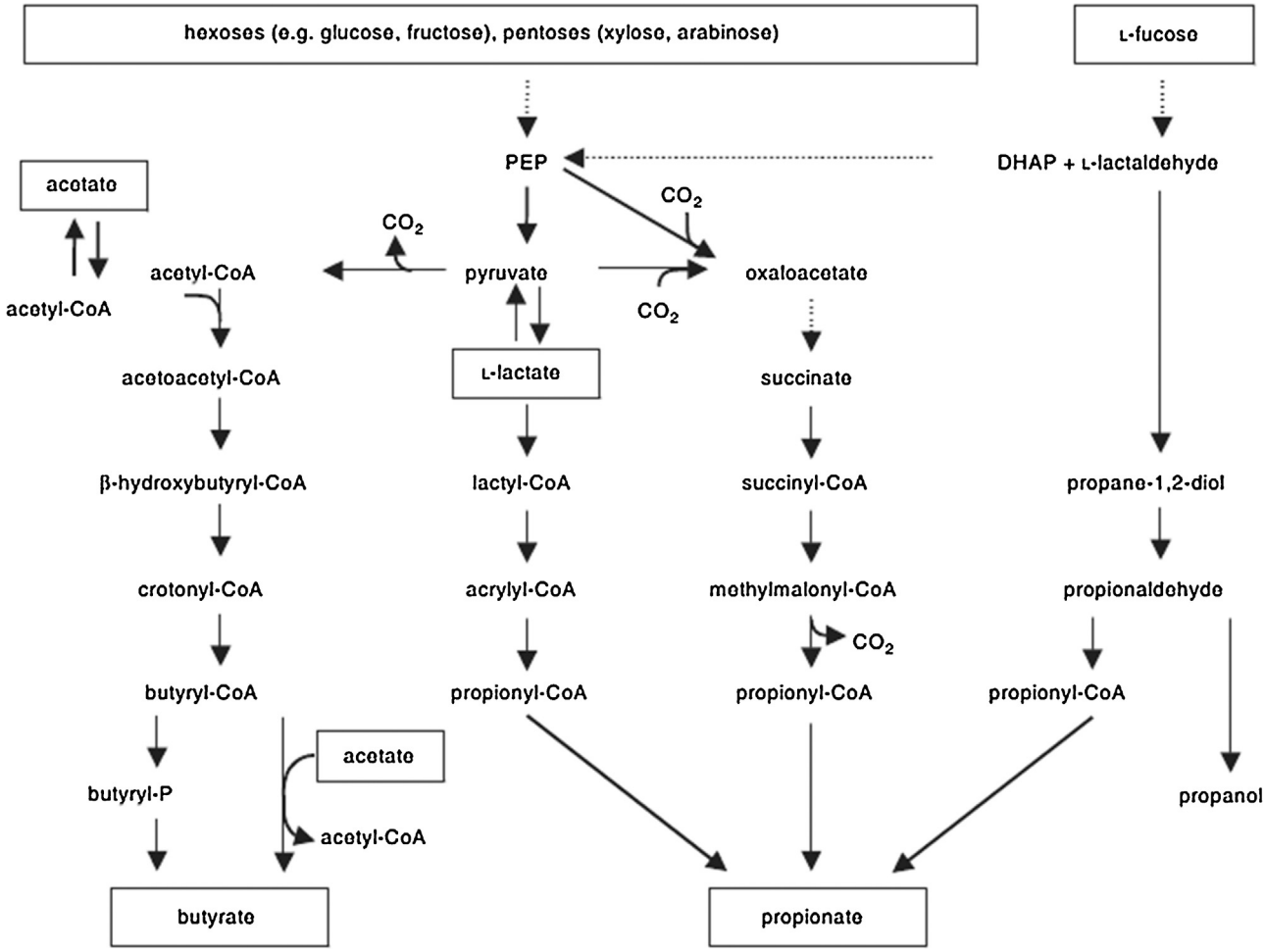

Fig. 3. Main fermentative reactions involved in the production of short-chain fatty acids [50].

acetyl-CoA. Hereafter, similar to the first pathway, hydrolysis can be used to form acetate from acetyl-CoA [83].

2.2.3.2. Propionate formation. Propionate can be synthesised following two different pathways. The first pathway has been addressed earlier in the paragraph regarding the use of primitive anaerobic electron transport chain. At low carbon dioxide partial pressures, succinate was converted into methylmalonate and then split into propionate and carbon dioxide. The generated carbon dioxide could then be reused for the carboxylation of phosphoenolpyruvate [80,81].

The second mechanism is based upon the acrylate pathway where pyruvate is firstly reduced to L-lactate by the amphibolic lactate dehydrogenase. It concerns a reversible reaction. In the reduction one molecule NADH is oxidised to NAD^+, after which L-lactate can subsequently be reduced to lactyl-CoA, acrylyl-CoA, propionyl-CoA and finally propionate [76].

It has to be noted that both pathways lead to more reoxidation of NADH to NAD^+ when compared to singular reduction of pyruvate to lactate.

2.2.3.3. Butyrate formation. The production of butyrate starts with the condensation of two acetyl-CoA molecules resulting in acetoacetyl-CoA. This molecule is then subsequently reduced to L (+)-hydroxybutyryl-CoA, crotonyl-CoA and finally butyryl-CoA [6,84]. From this molecule butyrate can be synthesised in two different ways. The first mechanism implies direct conversion of butyryl-CoA to butyrate using the enzymes phosphotransbutyrylase and butyrate kinase [85].

The alternative mechanism involves the use of butyryl-CoA and exogenously produced acetate that are converted into butyrate and acetyl-CoA by butyryl-CoA:acetate-CoA transferase [86].

2.2.4. Remarks

It should be noted that the addressed fermentative reactions do not necessarily occur in every fermenting micro-organism in the large intestine. Instead, it is more likely that just a few of these pathways are active per bacterial specie and that they are influenced by genetic factors and different environmental conditions of the large intestine [40]. The various influencing factors will be addressed later in this chapter.

2.3. Cross-feeding

Labelling studies using fermentation substrates that were labelled using radioactive isotopes have shown that metabolic intermediate and end products of primary fermenting bacteria can act as a fermentation source for other bacterial species. This phenomenon, involving bacterial species capable of fermenting dietary fibres providing substrates for other species present in the large intestine that are not or less able to ferment crude nutrients, is known as cross-feeding [39,82].

Cross-feeding allows growth and preservation of those bacterial species that are not capable to utilize crude nutrients that are

provided from the small intestine to the colonic gut flora [39,82]. Also, the complex interactions of the various fermentative reactions between bacterial species maintain a favourable fermentation environment in the lumen of the large intestine. In this way stacking of intermediate and end products, such as lactate, ethanol and succinate, as well as high partial pressures of various gasses is prevented, which ensures the continuity of fermentative reactions in the lumen. This is made possible by, for instance, the presence of methane-producing, nitrate- and/or sulphate-reducing as well as acetogenic bacterial species in the human colon [39,40,82].

For the utilization of released energy from the fermentative reactions reoxidation of NADH to NAD^+ is essential. The main applicable redox reaction is of endergonic nature in which released electrons are used for the reduction of hydrogen ions to hydrogen gas. This reaction requires a low H_2 partial pressure in the lumen. Accumulation of hydrogen gas can cause one of the major reoxidation reactions of NADH to stop, implying that released energy can not be used [79,82].

Stagnation is prevented by maintaining a low partial pressure, which is made possible by the ability of some bacterial species to reduce sulphate and nitrate [40,79]. These species can use anorganic anions as electron acceptors, enabling the exogenously formed hydrogen gas in the lumen to be used as an electron donor. Correspondingly, nitrate can be reduced to nitrite and sulphate to sulphide [40,84]. The use of hydrogen gas as an electron donor in these reactions prevents development of high H_2 partial pressures, thereby ensuring the continuity of the conversion reaction of NADH to NAD^+ by NADH:ferredoxin oxidoreductase. It appears that the nitrate- and sulphate-reducing reactions primarily occur when acetate is formed in relatively large quantities [74,87]. By isolating several bacterial species of the gut flora it was possible to investigate cross-feeding mechanisms between these species by means of studies using isotopically labelled substrates and the examination of various co-cultures [39].

Investigation of lactate-producing bacteria revealed that lactate is mainly synthesised by fermenting fructooligosaccharides and resistant starches [88,89]. By culturing these bacteria with *Eubacterium Halli* and *A. Caccae* butyrate was formed [90]. Several labelling studies have revealed that *E. Halli* and *A. Caccae* are able to convert L-lactate into acetyl-CoA, which is then exchanged for exogenous acetate [34,91]. In this way lactate can be utilised for the production of butyrate precursors [90]. Possibly, *E. Halli* features such a mechanism in order to avoid lactate accumulation in the large intestine. After all, *E. Halli* can comprise up to 4% of the total bacterial population of the large intestine [92].

Other research shows that various bacterial species belonging to the *Roseburia* genus are not able to convert lactate, fructooligosaccharides or resistant starches into butyrate. However, this does occur when the various species are co-cultured with *Bifido adolescentis*. The corresponding labelling study revealed that substrates are subjected to partial hydrolysis by *Bifido adolescentis*, after which the *Roseburia* species are able to ferment the resulting products to butyrate [39,93].

2.4. Influencing factors

A multitude of factors are of influence on the production pattern of short-chain fatty acids in the colon. Firstly, the supply of suitable substrates to the large intestine is of importance [11,40]. The substrates can then be degraded to various end products using several metabolic pathways [44]. However, certain substrate types give rise to specific dominant end products [40,44]. Accordingly, the fermentation of resistant starches results predominantly in the production of butyrate [16]. This is compliant with research using a human α-amylase inhibitor, which revealed that an increase of the butyrate production could be obtained when increasing the supply of starches [58]. In addition, the transit time of substrates through the intestinal system affects the level of butyrate production. Shorter transit times result in a larger butyrate output [94,95].

The production site also appears to be of influence on the production pattern and the quantities of produced short-chain fatty acids. Competition for fermentation substrates between bacterial species results in a concentration gradient of the produced end products across the large intestine. The beginning of the large intestine, the proximal area of the colon, features large quantities of substrates resulting in correspondingly large amounts of fermentation products. In accordance, the more distal areas of the large intestine feature smaller amounts of primary fermentation substrates which leads to lesser quantities of fermentation products [96].

However, dietary fibres that are not or only partially fermented are able to influence the production sites of several short-chain fatty acids. Application of difficult to ferment dietary fibres generally shifts the production sites to the more distal regions of the large intestine [97]. Well known is the use of psyllium, a hard to ferment dietary fibre, that shifts the butyrate production from resistant starches by increasing the bowel motility [98].

Combinations of substrates can have synergistic or antagonistic effects on the production of fermentation end products [33]. Combination of gum arabic and cellulose or pectins combined with guar gum both lead to an increase of butyrate production [33,99]. Hence, it can be deducted that the composition of human nutrition is of importance as it may lead to comparable effects [33].

Naturally, the physicochemical characteristics of the substrates involved in fermentative reactions are relevant as well. In particular the solubility of dietary fibres affects the production rate of end products. Freely soluble dietary fibres, such as pectin and guar gum, appear to be easy to ferment, whereas more slightly soluble dietary fibres, like cellulose and its derivatives, are harder to ferment [41].

Apart from the previously mentioned factors, Macfarlane and Macfarlane indicate several other host-specific influencing factors including age, neuroendocrine system activity, stress, pancreatic secretion and other secretions in the digestive tract, mucous production of the intestinal system, disease, drug use (especially antibiotics) and the epithelial cell turnover times [40].

Lastly, the microbial composition of the host's large intestine is of major importance. It shows strong interindividual differences and is subject to changes during the life of the host [40,100,101]. Exact charting of the microbiota of a host turns out to be very difficult using current cultivation techniques. The majority of the

Table 1
Identified microbial species and their main short-chain fatty acid products [102].

Acetate	Propionate	Butyrate	Lactate[a]
Bacteroides	Bacteroides	Roseburia	Bifidobacteria (L)
Bifidobacteria	Propionibacteria	Faecalibacteria	Bacteroides (D)
Eubacteria	Veillonella	Clostridia	Peptostreptococci (L)
Lactobacilli	Clostridia	Eubacteria	Lactobacilli (D/L)
Clostridia	Prevotella	Fusobacteria	Eubacteria (L)
Ruminococci	Porphyromonas	Peptostreptococci	Ruminococci (L)
Peptostreptococci	Megasphaera	Butyrivibrio	Fusobacteria (L)
Propionibacteria		Peptococci	Enterococci (L)
Veillonella			Clostridia (L)
Fusobacteria			Peptococci (L)
Butyrivibrio			Streptococci (L)
Peptococci			Enterobacteria (L)
Streptococci			Faecalibacteria (D)
Enterobacteria			Atopobium (L)
Atopobium			
Enterococci			

[a] L and D indicate L and D stereoisomers of lactate.

bacterial species in the colon concerns obligate anaerobes, that are not able to withstand aerobic environments [6]. However, it was possible to identify some relatively large bacterial populations, including their metabolic properties and corresponding end products [6,102]. Table 1 gives an overview of identified microbial species and applicable short-chain fatty acids.

Several analytical techniques were used for the identifications, including temperature gradient gel electrophoresis (TGGE) with 16S rRNA amplifications that were formed using polymerase chain reaction (PCR) techniques [101,103], restriction fragment length polymorphism (RFLP [104]) and various sequencing techniques [105,106]. However, the results obtained using these techniques are not easily reproducible. Using RFLP results in a vast interindividual diversity and variability of bacterial strains, as well as a strong influence of the moment of sampling. It is assumed that the observed variability is caused especially by differences in age and genotype of the testees [101].

One should realize that humans are not born with gut flora and no genes exist that code for hosting microbes in the intestines. Instead, a complex and diverse bacterial population develops during and after birth. The human foetal intestines in the uterus is sterile and only during birth colonisation starts due to maternal-foetal contact [107]. During the first five days after birth colonisation occurs following a more or less fixed pattern. In the first three days *Eneterobacteria* and *Streptococci* are predominantly present in the faeces featuring approximately 10^{11} cfu per gram faeces [108]. *Bifidobacteria* are present in detectable amounts after the second day and, in case of breastfeeding, become the dominant species four to five days after birth [34,109]. During weaning, implying the transition towards to a more adult diet, the gut flora's diversity increases [108]. Hence, infants feature a different production pattern of short-chain fatty acids when compared to the patterns of adults. Infants produce relatively low quantities of butyrate and much larger amounts of formate and ethanol [34].

3. Transport of short-chain fatty acids

Once short-chain fatty acids are synthesised by the microbial gut flora, these products become available for absorption by the intestinal epithelium. As has been indicated in the previous chapter, short-chain fatty acids are subject to oxidation by colonocytes for energy production. Physiological studies of ruminants have shown that short-chain fatty acids can be readily absorbed by the epithelial cells of their intestines [110]. Investigation of the absorption of acetate, propionate and butyrate in human epithelial cell lines also confirms that colonocytes can easily absorb these fatty acids across their apical membrane, after which a decrease of the intracellular pH can be observed. This is followed by a sodium-independent pH recovery [38,48,111–114]. Subsequently, the absorbed short-chain fatty acids are metabolised into glucose, ketone bodies and amino acids [12,115]. In this process the substrates undergo multiple oxidations forming strongly reduced compounds. Colonocytes appear to prefer the oxidation of butyrate into CO_2 to the oxidation of propionate and acetate, independently of the presence of other short-chain fatty acids [36]. However, epithelial metabolization of the short-chain fatty acids is incomplete, which implies that some of these fatty acids are able to pass through the basolateral membrane, whereupon they can reach the liver and the systemic circulation [12,22,115–117].

In the past 35 years a lot of research has been performed regarding colonic transport mechanisms of short-chain fatty acids. Charting these mechanisms is hampered by two different aspects of the fatty acids. Firstly, short-chain fatty acids form a major substrate for the metabolism of colonocytes, which complicates investigation of applicable transporters. To circumvent this problem, many studies use short-chain fatty acids that are metabolised to a lesser extent, such as propionate and isobutyrate. Another problem is the existence of equilibria between the luminal, basolateral and intracellular compartments of the intestinal epithelium. Short-chain fatty acids are weak acids, implying equilibria between the protonated and deprotonated form in all these compartments. This means that small changes in pH strongly affect the relative distribution of short-chain fatty acids and consequently hinders investigation of absorption mechanisms [110].

In this chapter the mechanisms underlying the transport of short-chain fatty acids across cell membranes of the colonocytes will be addressed.

3.1. Apical transport

In vivo research has shown that short-chain fatty acids are easily absorbed at the apical membrane, in which the absorption approximates the colonic sodium absorption (300 mmol), hence presumably nearing the maximum absorptive capacity for short-chain fatty acids [110]. It is assumed that two separate transport mechanisms are involved in the apical absorption of these acids. The fatty acids are weakly acidic compounds featuring a pK_a value of approximately 4.8. The pH value in the lumen of the large intestine ranges from 5.5 to 6.5. This implies the existence of an equilibrium between the protonated and deprotonated state of the short-chain fatty acids, wherein 90–99% of the compounds will be present in the deprotonated form. The protonated form is uncharged and non-polar allowing easy diffusion through colonic cell membranes. The deprotonated form carries a negative charge and is therefore unable to pass the lipophilic cell membranes, thus requiring absorption by transporters [110]. Research has found that the neutral, protonated form is subject to non-ionic diffusion in the weakly acidic environment of the lumen [118–121].

The second transport mechanism deploys integral transmembrane proteins, which act as transporters for the negatively charged, deprotonated fatty acids. Several research groups were able to identify various anion transporters for the absorption of short-chain fatty acids involving carrier-mediated transport from the lumen [121–124].

3.2. Non-ionic diffusion

As has been indicated before, short-chain fatty acids are weak acids featuring a carboxyl group with a pK_a value of about 4.8. This means that only a small fraction will be protonated and thus will be available for non-ionic diffusion in the lumen of the large intestine that has a pH value ranging from 5.5 to 6.5. For this type of transport it is essential that fatty acids are present in the neutral, protonated form in order to diffuse through the lipophilic, apical membrane of the colonocytes [110]. This also means that the absorption of the protonated short-chain fatty acids improves with an increase of the chain length these fatty acids as the non-polar fraction of these molecules increases correspondingly [125]. In spite of the strong concentration gradient that exists between the lumen and the serosa of the colon, paracellular transport hardly occurs. For this reason, research was mainly conducted to the mechanisms concerning transcellular transport by means of non-ionic diffusion [110]. In investigations using acidified mucosa samples from sheep and rabbits a larger absorption of short-chain fatty acids could be observed. Therefore, it appears that short-chain fatty acids predominantly diffuse to compartments in which they are more ionized due to higher pH values [120].

Hence, transcellular transport by means of non-ionic diffusion requires short-chain fatty acid molecules to be present in the protonated form. The intestinal epithelium is able to deliver protons to the lumen of the large intestine enabling protonation of the fatty

acids. The proximal region of the colon is rich in sodium-hydrogen antiporters that facilitate the epithelial release of protons. However, protons can also be generated by the epithelium via hydration of CO_2 into H_2CO_3, a weak acid, using the carbonic anhydrase enzyme [38].

It appears that an increase of released protons by the intestinal epithelium can result in stronger absorption of fatty acids. Accordingly, stimulation of the sodium-hydrogen antiporters in the proximal region of the intestine results in an increased acidity of the lumen as well as a larger absorption via non-ionic diffusion. Also an opposite effect was observed in the research. Inhibition of colonic sodium-hydrogen antiporters with amiloride or theophylline leads to a reduced absorption of short-chain fatty acids [126]. A similar relation could be observed with stimulation or inhibition of the H^+/K^+ ATPase in the distal regions of the colon [124].

3.3. Transporters

In addition to the uptake through non-ionic diffusion, the colonocytes are also capable of absorbing the deprotonated and thus negatively charged fraction of short-chain fatty acids by using transmembrane proteins [121–124]. In molecular characterisation studies particularly plasma membrane vesicles from both the apical and basolateral membrane were investigated [127,128]. From these studies it was possible to identify three different transmembrane protein families, which contribute to the uptake of anionic short-chain fatty acids.

3.3.1. $SCFA^-/HCO_3^-$ transporter

In perfusion studies of the colon, wherein short-chain fatty acids were administered, a relation was found between luminal secretion of bicarbonate (HCO_3^-) and the administration of these fatty acids [38,129–131]. Further research into the short-chain fatty acid-dependent bicarbonate secretion revealed that it concerns most likely an apical $SCFA^-/HCO_3^-$-exchange, that predominantly takes place in the superficial cells of the colon [132,133]. This first anion-transporter family has been further examined using apical membrane vesicles (AMV) and was shown to occur on the apical membrane of the human ileum and colon. The transporters are antiporters that link the uptake of short-chain fatty acids to the secretion of bicarbonate into the lumen of the large intestine [127,128]. The absorption of short-chain fatty acids by the antiporter was shown to be independent of the Cl^-/HCO_3^- antiporter of the colonocytes and of both the luminal and intracellular sodium concentration [123,124,127,128,134]. In particular propionate and butyrate were found to be taken up well by a Na^+-independent $SCFA^-/HCO_3^-$ transporter [127,128].

It has to be noted that molecular identification of the antiporter is not easy. For a complete characterisation the substances that may inhibit or compete the transporter need to be identified, as well as the concentrations of these substances at which these effects occur and above all the results of such investigations must be reproducible [110]. Research into inhibition of this transporter, using the universal anion-transporter inhibitor *4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS)*, revealed in a number of studies an inhibitory effect at high concentrations of this inhibitor. However, this effect was not always observable and reproducible [110,132].

However, short-chain fatty acids proved to be able to compete their mutual absorption by colonocytes with the aid of this transporter. For example, the short-chain fatty acid mercaptopropionate is capable of inhibiting the absorption of butyrate in the distal colon of rats. It should however be noted that mercaptopropionate affects the intracellular acidity differently than other short-chain fatty acids, so it is not clear whether the inhibition is due to the changed degree of intracellular acidity or to this specific competition [110,134].

3.3.2. Monocarboxylate transporter

The second type of transporter constitutes a family of transporters for monocarboxylates and is synthesised from the SLC16 gene family [135,136]. The uptake of short-chain fatty acids from the lumen is possible as the transporter catalyses co-transport with hydrogen ions (H^+). The monocarboxylate transporter (MCT) acts as a symporter and enables electroneutral absorption of the negatively charged, deprotonated fatty acids as the negative charge is compensated by co-transport with this cation [137]. Apart from short-chain fatty acids, these transporters can also transport lactate and pyruvate [138,139]. To date, fourteen isoforms of the monocarboxylate transporter have been identified [140].

Apical transport of butyrate in Caco-2 cells, a human colorectal cancer cell line, involves isoform 1 of the transporter family (MCT1) and exhibits typical Michaelis-Menten kinetics. The molecular transport mechanism of MCT1 in the apical membranes of enterocytes has been described with the aid of kinetic analyses [141]. It has been found that binding of the hydrogen ion (K_m 0,2 μM) occurs first, after which the substrate, for example lactate (K_m 4–7 μM), binds. Conformational change follows, causing translocation of the substrates through the membrane. Subsequently, the bonds with the substrate are broken and the binding sites are moved to the opposite side of the membrane. This recovery step takes longest and is therefore the rate limiting step for the transport of lactate through the membrane, as could be observed in trans-stimulation experiments [137]. Lowering the luminal pH or increasing the intracellular pH results in an increase in the V_{max} by reducing the time that is required for reorientation of the transporter [7,128,142].

Investigation on possibilities for inhibition of monocarboxylate transporters showed that insensitivity to inhibition by DIDS exists [141]. In contrast, MCTs could be inhibited by 4-hydroxy cinnamate (4-CHC) [7,141]. Studies with radioactively labelled [14C]-butyrate revealed that the short-chain fatty acid transport activity of MCT1 could be inhibited by applying 4-CHC. It was also discovered that the absorption of butyrate by MCTs is independent of the sodium concentration in the intra- and extracellular compartment, but is dependent on time and the acidity of these compartments [141]. Research with antisense DNA has demonstrated the involvement of MCT1 in the apical transport. Transfection of Xenopus oocytes with cDNA for MCT1 revealed an inhibition of the butyrate uptake. A similar study involving Caco-2 cells that were transfected with cDNA showed a 50% decrease of butyrate absorption [141]. Inhibition with antisense DNA is based on the formation of complementary microRNA fragments, which are capable of binding the MCT1 mRNA to form a piece of double-stranded RNA. The double-stranded RNA is then degraded intracellularly, causing inhibition the gene expression. Apart from inhibition of the butyrate uptake by the administration of inhibitors, the absorption of butyrate by MCTs is also subject to inhibition due to competition with other substrates. Both lactate and pyruvate, which are substrates of MCT as well, are able to inhibit the butyrate absorption effectively by competing for transport by MCT1 [141,143].

As mentioned earlier, until now fourteen different genes in the SLC16 gene family have been identified. From many of these genes the corresponding gene product, a specific isoform of the monocarboxylate transporter, was identified and its distribution through the human body could be assessed. MCT1 (SLC16A1 gene) was the first isoform that could be successfully cloned from Chinese Hamster Ovary (CHO) cells and wherein it was shown that this transporter was capable of transporting monocarboxylates, pyruvate and lactate [140,144]. MCT1 is involved in the uptake of short-chain fatty acids at the apical side of human colonocytes, but can also be found in the heart, red muscle tissue and erythrocytes [7,141,143,144]. The molecular identity of MCT1 was assessed using specific labelling studies of erythrocytes of rats and rabbits [145],

whereafter by means of purification and N-terminus sequencing a protein of 40–50 kDa could be identified [146]. This terminus is identical to a 12 TMD-transporter (MEV), which was previously cloned by *Goldstein* and *Brown* from a mutant CHO cell line, which showed enhanced mevalonate absorption [147].

However, MCT2 and MCT3 (SLC16A7 and SLC16A8 respectively) feature a more limited distribution throughout the body [148–151]. Isoform 2 was initially found in the parietal cells of the stomach of hamsters through *in situ* hybridisation [135] and PCR (polymerase chain reaction) analysis and was found not to be expressed in the intestinal tract of hamsters and humans [138,140]. Furthermore, MCT2 is strongly expressed in the liver [152]. The expression of MCT3 is even more limited and is found in very low concentrations on the membranes of colonocytes. This isoform also occurs at the pigmented epithelial layer of the retina, where it provides an efflux of lactate towards the choroids surrounding the eye [150,151].

Isoforms 4 and 5 of the MCTs (SLC16A3 and SLC16A4 respectively) appear to be present on the membranes of human colonocytes. In addition, both transporters exist on skeletal muscle tissue and in the placenta enabling local release of lactate from the muscles and the fetus [140,153,154].

MCT isoform 6 (SLC16A5) is especially found in the liver and also in lower concentrations in the placenta. Research into the MCT7 (SLC16A6) expression yielded that this isoform is predominantly expressed in the brain and the pancreas. The expression of isoform 8 (SLC16A2) is restricted to the liver, the kidneys and the myocardium [153].

Hitherto, the expression patterns of the remaining members of the SLC16 gene family, such as MCT9, -11, -12, -13 and -14 (SLC16A9, -11, -12, -13, -14 respectively), have not been established. These isoforms have only been identified using analyses from various human genomic expressed sequence tag (EST) databases [139,140].

Also, a lot of research into the expression patterns of various colonic cell lines has been conducted. Immunohistochemical studies of cecal cells from hamsters revealed that MCT1 is particularly present on the basolateral membrane. Accordingly, this was also observed in jejunum cells of rats, but here the expression of MCT1 was found to be shifted towards the apical side in the more mature surface cells [155]. Several western blot studies have demonstrated that MCT1, -4 and -5 are expressed strongly on colonocytes in the more distal parts of the human colon and to a lesser extent, but still in significant concentrations, in the ileum and proximal portions of the colon [156]. In addition, Ritzhaupt *et al.* demonstrated by means of western blotting that MCT1 expression is restricted to the luminal side of the colonocytes of pigs. Buyse *et al.* confirm this phenomenon and revealed the expression of apical MCT1 in the human colorectal cancer cell line Caco-2 using immunofluorescence techniques [156–158]. Research by immunoblotting and immunohistochemistry studies into human colonocytes further indicate that the expression of MCT4 and -5 is primarily confined to the basolateral membrane of the colonocytes [156]. Additionally, a weak MCT3 expression was measured on the basolateral side of the colonocytes. However, here MCT3 was expressed only in low concentrations. The determination required a twenty-fold higher concentration of antibody for this particular isoform relative to the other MCTs in order to enable detection of the expression [156]. A stronger expression of MCT3 could be observed in the pigmented epithelial layer of the retina, where it provides the release of lactate to the choroids of the eye [150,151].

A deviant expression pattern was observed in the colorectal cancer cell line Caco-2. Research using reverse transcriptase-polymerase chain reaction (RT-PCR) revealed that isoforms MCT1, -3, -4, -5 and -6 were detectable on the membranes of these colonocytes [135,153]. The expression of MCT6 appears to be limited to this carcinoma cell line and is not observed in studies into the expression patterns of healthy colorectal cell lines [156]. The simultaneous expression of different MCT isoforms on the intestinal epithelium hampers research on differences in the molecular mechanisms of these isoforms and further molecular identification of the various transporter complexes for short-chain fatty acids [156]. Furthermore, one may wonder why multiple isoforms are expressed in colonocytes. A possible explanation for this phenomenon lies in potential differences in kinetics for the different short-chain fatty acids. In this way the concurrent expression of multiple isoforms could facilitate efficient transport for the various fatty acids. Alternatively, some of these isoforms could be involved in housekeeping of the basic cellular functions [156].

Another explanation for the simultaneous occurrence of the isoforms are differences in the regulation of these various forms of MCT. In fact, expression of MCT6 could only be observed in the Caco-2 cell line using RT-PCR [153]. Possibly, modification of the environment of the intestinal tract leads to changes in the expression patterns of colonocytes. Consequently, an inflammatory environment as a result of inflammatory reactions in the gut might lead to a modified expression of different isoforms [156].

Short-chain fatty acids are also able to stimulate the luminal, electroneutral absorption of sodium by colonocytes [133,159–161]. The stimulating effect is particularly apparent in the proximal region of the colon, where the largest quantities of short-chain fatty acids are produced by fermentation of dietary fibres by the intestinal flora. The electroneutral uptake is the main absorption route of sodium in the colon and makes use of different sodium-hydrogen exchangers (NHE) that exchange intracellular hydrogen ions for luminal sodium, which enables recovery of the acidity of the intracellular environment [160,162]. Isoforms 2 and 3 are both expressed on the apical membrane of the colonocytes, but are regulated in different ways and under some circumstances, there seems to be a reciprocal relationship between these two isoforms [163]. NHE isoform 1 is present in lower concentrations on the basolateral membrane and therefore does not contribute to the luminal sodium uptake [164]. It appears that short-chain fatty acids are able to modulate the expression and activity of the apical-bound sodium-hydrogen exchangers [165].

Research into the stimulating effect of short-chain fatty acids on the human colonic epithelial cell line C2/bbe (C2) monolayers indicates that only the activity of NHE3 can be increased [160]. The activity and expression of NHE2 in the colonocytes thereby appears unchanged and therefore not affected by these fatty acids [160]. Also, short-chain fatty acids do not need to be metabolised in order to stimulate the sodium uptake by NHE3. Accordingly, research conducted using the poorly-metabolisable isobutyrate resulted into comparable effects [160].

The induction of NHE3 may be caused by an increase in the intracellular acidity caused by the absorption of short-chain fatty acids, as was observed with isolates of colonic crypts of Lieberkühn [159,161,166]. This was also observed in a study in which acidosis was induced in a renal cell line of the opossum kidney. As a result of the low intracellular pH value, a chronically increased expression and activity of NHE3 was observed [167]. It is thought that as a result of the increased intracellular acidity NHE3 is induced by a c-src pathway, as addition of a c-src inhibitor decreased this stimulation [168]. The monocarboxylate transporters may contribute to the stimulation of NHE3. After all, the symporter transports anionic short-chain fatty acids along with protons to the intracellular environment, wherein the protons may cause an increase of the intracellular acidity.

Possibly, short-chain fatty acids can affect the transcription of NHE3 themselves after being absorbed. In the human DNA several

butyrate response elements (BRE) have been identified, that may be important for the regulation of the expression of different genes [169,170]. In the human NHE3 gene, at least three potential butyrate response elements have been identified in the 2-kilobase 3′-flanking promoter regions. However, it is not yet clear whether these elements are involved in the observed induction of NHE3 in colonocytes [160].

3.3.3. Sodium-dependent monocarboxylate transporter

The final transporter that contributes to the apical uptake of short-chain fatty acids is SMCT1. In 2004, it was discovered that gene SLC5A8, which belongs to the solute-linked co-transporters SLC5 gene family, encodes for a Na^+-coupled symporter for short-chain fatty acids lactate, pyruvate, acetate, propionate and butyrate [171–174]. Gene products belonging to this family transport substances including glucose, *myo*-inositol, iodide, choline and B-vitamin complexes [175]. The substrate specificity of SLC5A8 shows a strong resemblance to that of MCT [140], but in contrast to the latter transporter, SMCT1 co-transport involves sodium ions rather than protons. This difference has implied that the protein product encoded by the SLC5A8 gene is known as the sodium-dependent monocarboxylate transporter 1 (SMCT1) [176].

An additional difference between the SMCT1 and the short-chain fatty acid transporters from the SLC16 gene family is that SMCT transporters feature electrogenic instead of electroneutral transport. The transport process of anionic fatty acids is linked to the co-transport of Na^+ with a stoichiometry of 1–2. As a result of the net charge transfer across the membrane, this process is electrogenic and driven by an electrochemical Na^+ gradient over the cell membranes [176].

In addition, it has been possible to identify a second form of SMCT, which is synthesised from gene SLC5A12. This second isoform, SMCT2, shows 59% similarity with SMCT1 at amino acid level. SMCT2 has an similar substrate profile to that of SMCT1 and is also dependent on co-transport of sodium ions for its transport activity, but is found to have a much lower affinity for its substrates [177].

Other differences include the sites where the two isoforms are expressed. With the help of RT-PCR studies it was possible to map the distribution through the body. Northern blot analysis of mRNA coding for SMCT1 (SLC5A8) revealed strong expressions in the small intestine, colon, thyroid and kidney [171,178]. The expression level of SLC5A8 mRNA was found to be higher as the regions of the large intestine were located more distally, which is in agreement with previous RT-PCR studies of micro-dissected epithelial cells of mice [171,179].

Northern blot studies using mRNA from SLC5A12 (SMCT2) showed a different distribution. Expression of this transporter was observed in the kidneys, skeletal muscles and small intestine, but was absent in the distal parts of the intestinal tract [177].

It was also found that the physiological function of the transporter differs per tissue. In the large intestine the expression of SMCT1 enables the absorption of mainly butyrate and propionate and also provides the active reabsorption of lactate and pyruvate in the kidneys [171,174,176]. In the thyroid the expression of SMCT1 possibly enables the transport of iodide. For the formation of thyroid hormones, it is essential that iodide is transported through the apical membrane to the colloid lumen of the thyroid follicular cells. Here, iodide reacts with the tyrosine residues in thyroglobulins to form such thyroid hormones [180]. As pyruvate and lactate are also formed during the production of thyroid hormones, these can inhibit iodide transport as a competitive substrate and possibly regulate the production of thyroid hormones by limiting the influx of iodide into the colloid lumen [176].

Furthermore, simultaneous expression of different isoforms can be of importance for the functioning of various organs, as can be exemplified by the kidneys. In this organ, the SMCT2 transporters with lower affinity are mainly present in the proximal S1 portion of the tubules, whereas SMCT1 transporters with a relatively higher affinity are expressed in the more distal regions of the nephron. In this way, effective reabsorption of lactate and other substrates by these transporters is maintained, despite the decrease of the substrates concentrations in the lumen of the more distally located regions of the nephron by prior, proximal reabsorptive processes [177,181].

Through research by Li *et al.* SLC5A8 has been identified as a potential tumour suppressor gene and inhibition of the expression of this gene was found in approximately 60% of primary colorectal cancers. The silencing of this gene is caused by strong methylation of a CpG site in the DNA, which regulates the expression of SLC5A8 [171–173,182].

3.4. Basolateral transport

The fraction of the short-chain fatty acids that is not metabolised by the colonocytes after apical absorption can leave the colonocytes at the basolateral side and reach the portal vein [12,22,115–117]. Because the intracellular pH value is much higher than the pH value that prevails in the lumen of the large intestine, absorbed short-chain fatty acids, having carboxylic acid group featuring a pKa value of around 4.8, are present in the deprotonated, negatively-charged form. Since the molecules in this form are strongly polar, no diffusion can take place through the lipophilic membrane, which implies that basolateral transport of the fatty acids requires the use of transporters [110].

Research using basolateral membrane vesicles (BLMVs) from rat colonocytes has demonstrated the existence of a basolateral $SCFA^-/HCO_3^-$ transporter [183]. The transport process of the anion symporter is dependent on the pH gradient and the intracellular bicarbonate concentration and shows a transport direction opposite to that of the apical $SCFA^-/HCO_3^-$ transporter [110,117]. Research indicates that the receptor can be inhibited by 1 mM nuflumic acid, but is insensitive to inhibition by stilbene derivatives, DIDS, SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate) and acetazolamide [117]. However, these results are not consistent; research on this transporter by Reynolds *et al.* did actually show inhibition of the transporter on the basolateral BLMVs when applying 1 mM DIDS and acetazolamide. Possible explanations for the inconsistent results are differences in the cell lines used or the occurrence of MCTs on the basolateral membrane that are insensitive to inhibition by DIDS [141].

Also, it is likely that the $SCFA^-/HCO_3^-$ transporter found on the basolateral membrane differs from the transporter that is present on the apical membrane of the colonocytes. Kinetic analysis of the uptake activity of butyrate by the two transporters using a Lineweaver–Burk plot provided K_m values of the apical and basolateral transporter being $1.5\,mM \pm 0.2\,mM$ and 17.5 mM, respectively [117]. Further characterisation studies are required in order to identify molecular differences.

Using immunoblotting, the presence of MCT isoforms 3 (SLC16A8), 4 (SLC16A3), and 5 (SLC16A4) could be demonstrated on the basolateral membrane of colonocytes. However, MCT3 is only present in such low concentrations on the basolateral membrane that it is unlikely that this isoform significantly contributes to the transport of short-chain fatty acids into the portal vein [156].

Fatty acids and protons are transported by MCT4 on the basolateral membrane in the opposite direction relative to that of MCT1. Similar to MCT1 on the apical membrane, this transporter uses electroneutral transport and thereby depends on cationic hydrogen ions for co-transport. However, this transporter has a lower affinity for these substrates than MCT1 [140].

MCT5 also appears on the basolateral membrane of colonocytes, but it is unclear whether this transporter is capable of transporting short-chain fatty acids through this membrane. For clarification further molecular identification of this isoform is necessary [139,156].

4. Metabolism and excretion of short-chain fatty acids

Short-chain fatty acids are metabolised at three different locations in the body [184]. First of all, the fatty acids that are absorbed by the colonocytes are partly used for the production of energy [110,184]. However, the metabolism of these cells is incomplete and a fraction of the fatty acids will therefore have the opportunity to pass through the basolateral membrane in unaltered form [12,22,115–117]. After passing this membrane, these substances will enter the portal vein, which flows into the liver. Entry of the short-chain fatty acids into systemic circulation requires passage through the liver in unmetabolised form. Then, the peripheral muscle tissue can metabolise these unaltered fatty acids for energy production [184].

However, the liver is specialised in converting endo- and exogenous substances into easily excretable compounds. To this end, two different reactions occur in the hepatocytes of this organ. The first type of reaction is known as Phase I reactions and makes use of a highly diverse and complex system of cytochrome P450 enzymes. This enzyme system handles the biotransformation of a wide variety of substances [185,186].

The second type is known as phase II reactions and is based on the conjugation of, whether or not biotransformed, conjugates with anionic substrates, such as sulphate, glutathione, glucuronate, and other anionic, polar groups. The transformed compounds can then be released by the liver into the bile, whereafter they leave the body through the stool. Another option is the release of the metabolites into the systemic circulation, after which they become available for excretion through the kidneys [185,186].

First, the substrates must be absorbed by the hepatocytes in order to facilitate these metabolic reactions. To this end, they must pass through the sinusoidal membrane of these cells. [185,186] Recently, two different transporters have been identified on this membrane which facilitate the absorption of fatty acids propionate and butyrate; OAT2 and OAT7 respectively [22,187]. Both transporters belong to the sodium-independent, multi-specific organic anion transporters (OAT), a subgroup consisting of eight isoforms of the solute carrier family 22 (SLC22) gene family [188,189]. The SLC22 gene family comprises several plasma membrane proteins, including organic cation transporters (OCT), organic zwitterion/cation transporters (OCTN), wherein in particular the organic anion transporters are involved in the hepatic and renal excretion. Additionally, the OAT-subfamily plays a major role in renal reabsorption and distribution of organic anions through the body, in drug–drug interactions and toxicity of anionic substances, such as uremic toxins and nephrotoxins [22,190].

First the characteristics of the two different transporters will be discussed. Thereafter, the metabolic capacity of the hepatocytes on the degradation of short-chain fatty acids and the way in which the absorbed fatty acids are metabolised will be addressed. Finally, the excretion routes of the metabolic end-products are discussed.

It should be noted that there is still a lot of research conducted into the transporters involved in the uptake of short-chain fatty acids. Only recently the two aforementioned plasma membrane proteins have been identified [22,187]. Other research shows that fatty acid acetate may be absorbed from the portal vein by the hepatocytes, but the mechanism has not been elucidated as of yet [191].

4.1. Organic anion transporter 2 (OAT2)

The second isoform of the organic anion transporters is synthesised from the SLC22A7 gene and is strongly expressed in the liver. OAT2 is also, to a lesser extent, present in the kidneys where the transporter is expressed on the basolateral membrane of the proximal tubule cells and provides the secretion of urate [187,192]. The transporter has a very diverse substrate pattern, and is capable of transporting organic anions from both exogenous and endogenous source. For example, OAT2 is able to transport several compounds such as glutarate [193], urate [194], L-ascorbate, orotic acid, cAMP, prostaglandins E_2 and F_2 and α-ketoglutarate into the cells [192,195]. In addition, the transporter is involved in the uptake of various drugs including salicylate, bumetanide, erythromycin, tetracyclines, zidovudine, ranitidine [196], methotrexate, allopurinol, and 5-fluorouracil [192,193,195,197,198].

Research into the transport mechanism indicates that OAT2 concerns an antiporter, which facilitates the exchange of intracellular dicarboxylates for extracellular organic anions. The transport process is therefore electroneutral, wherein the absorption is driven by an outward dicarboxylate gradient across the sinusoidal membrane [192,193,198,199]. It also appears that the transcription of the gene encoding OAT2, SLC22A7, can be activated by hepatocyte nuclear factor 4α (HNF-4α). This nuclear transcription factor has a binding motif in a direct repeat element in the 5′-flanking region of the gene and is able to initiate the transcription of the gene by binding this element [200].

With the aid of Xenopus oocytes expression systems as well as labelling studies, in which different fatty acids were labelled with tritium, the ability of OAT2 to absorb fatty acids was investigated. The oocytes were transfected with an OAT2 gene derived from mice, after which the oocytes were exposed to a propionate-rich medium. The oocytes that were transfected with mOAT2 absorbed significantly more propionate in comparison to water-injected oocytes, which shows that mOAT2 facilitates the uptake of propionate. Therefore, it is probable that the human form of OAT2 (hOAT2) facilitates the absorption of this short-chain fatty acid in the liver in humans [22].

The labelling studies showed that the mOAT2 transfected oocytes were not able to transport fatty acids butyrate, valerate and octanoate, when exposed to these substances. It is suspected that these substances are able to bind to the substrate binding site, but that no subsequent translocation occurs [22].

4.2. Organic anion transporter 7 (OAT7)

OAT7 is synthesised from SLC22A9 gene and differs in some aspects from the other isoforms of the OAT-subfamily. This isoform of the receptor is only expressed in the liver, in contrast to the other isoforms which are also, whether or not in a slight degree, present in multiple tissues [188,189]. It appears that the transcription of the SLC22A9 gene can be activated by hepatocyte nuclear factor 1α (HNF-1α), which can bind in the promoter region of the gene, fourteen nucleotide bases away from the transcription start site [201]. OAT7 as well as OAT3 and OAT4 prove to be able to transport sulphate conjugates of steroid hormones and xenobiotics. However, OAT7 exhibits a much stronger preference for the transport of these sulphate conjugates as compared to the other two isoforms, as could be learned from studies in which Xenopus oocytes were transfected with OAT7 [187,202,203]. The differing substrate specificity is further confirmed by research into the inhibition of this transporter, which shows that OAT7 can not be inhibited by probenecid, an inhibitor that was found to be able of inhibiting the other members of the OAT-subfamily [187].

The absorption of organic anions by the transporter takes place by transporting sulphate conjugates of the steroid hormone

estrone, one of the estrogenic hormones, in the opposite direction. The antiporter of this transmembrane protein is dependent upon the intracellular sulphate conjugation of estrone by the hepatocytes [187,204]. Here, the estrone sulphate is formed by cytosolic sulfotransferase and is present in concentrations ten-fold higher than that of the unconjugated steroid hormone [205]. The conjugated estrone molecules feature a longer half-life and probably represent the precursors for the synthesis of steroid hormones by steroid-sensitive tissues. The hepatocytes thereby act as a reservoir for the conjugates of these hormones [206].

The transporter is expressed on the sinusoidal membrane of hepatocytes and appears to be able to take up butyrate from the portal vein whilst exchanging estrone sulphate. This transport is bi-directional, which implies that the transport direction of the two substrates of the membrane transporter can be reversed, depending on the metabolic status of the short-chain fatty acids in the liver. Because of the role of hepatocytes in the metabolism of steroid hormones and butyrate, under physiological conditions predominantly the absorption of butyrate will occur. OAT7 thereby forms the main route of butyrate uptake in the liver [187].

However, labelling studies revealed that the estrone sulphate-mediated uptake of butyrate by OAT7 could be inhibited by different short-chain fatty acids featuring three to five carbon atoms [187]. It possibly concerns a competition for the substrate-binding site of OAT7 between butyrate and these other short-chain fatty acids, whereby translocation only occurs in the case that butyrate binds at this site.

In addition, the binding affinity of OAT7 to estrone sulphate was investigated in kinetic studies. The K_m values for estrone sulphate were compared between Xenopus oocytes transfected with OAT7 and the Schneider 2 mammalian cell line (S2 hOAT7) and amounted 8.7 μM, and 40.7 μM, respectively. This difference in affinity is probably caused by differences between the expression systems used. Research on the affinity of OAT2 for estrone sulphate using S2-hOAT2 cells did not yield reliable results. The K_m value appears difficult to determine due to the very low affinity of the transporter for estrone sulphate [189].

4.3. Metabolism by the liver

The three short-chain fatty acids that can evade the metabolism of the colonocytes of the large intestine to a significant degree and reach the portal vein are acetate, propionate and butyrate. Subsequently, these fatty acids will have to pass through the liver in order to enter the systemic circulation. During the passage of the liver, these substances are metabolised to various degrees by the hepatocytes.

Acetate in the systemic circulation has two origins. Exogenous acetate is formed in the fermentation of dietary fibre by the bacteria present in the colon. Moreover, endogenous generation of acetate occurs in different tissues and organs, after which the produced acetate is released into the blood [207,208]. Research has shown that the liver is capable of metabolising approximately 70% of the supplied acetate to other substances [191]. The hepatocytes use the acetate not only as an energy source, but also as a substrate for the formation of acetyl-CoA from acetoacetate, long chain fatty acids and β-hydroxybutyrate [209]. It is also used as a co-substrate for the formation of glutamate and glutamine. The remaining acetate is oxidised in the periphery, such as the adipose tissue, the heart, the kidneys and muscle tissue [191,208]. In the presence of ethanol in the blood, it appears that less oxidation of acetate occurs in the hepatocytes and degradation mainly takes place in the peripheral tissues [210].

In order to avoid high, possibly toxic concentrations of propionate and butyrate in the systemic circulation, the liver clears a large portion of these fatty acids [211,212]. Concentration measurements of propionate in the portal and hepatic vein indicate that approximately 30% of the propionate is taken up by the liver. The unaltered portion is then extensively metabolised in the periphery. Assessments of the propionate concentrations in the peripheral veins yielded values that were found to be 23% lower than the concentrations that were measured in the hepatic veins [16,191]. Propionate features an odd number of carbon atoms and is therefore oxidised to propionyl-CoA by propionyl-CoA synthetase in hepatocytes. This oxidation product is then subsequently converted in three steps to succinyl-CoA by proprionyl-CoA carboxylase, methylmalonyl-CoA epimerase and methylmalonyl-CoA mutase. Succinyl-CoA can then participate in the citric acid cycle, in which oxaloacetate is formed. Oxaloacetate can be converted by the liver into glucose and can therefore contribute to gluconeogenesis in this organ as a precursor molecule [21,22,213,214].

As butyrate is preferred by the colonocytes for their metabolism, only a small fraction of the butyrate produced in the colon is able to leave the colonocytes through the basolateral membrane. As a consequence, butyrate is only found in low concentrations of around 28.8 μM in the portal vein [213]. As stated earlier, both butyrate and propionate may be toxic, when they exist in high concentrations in the systemic circulation [211,212]. The liver appears to be able to absorb and oxidise virtually all of the butyrate from the portal vein, causing low butyrate concentrations in the systemic circulation. In the hepatocytes the butyrate molecules are first converted to butyryl-CoA by butyryl-CoA synthetase and then to acetyl-CoA [21,22].

The acetyl-CoA molecules, which are produced by the hepatic oxidation of acetate and butyrate, can participate in the citric acid cycle to generate NADH and ATP [21]. During fasting, acetate and butyrate can be used for the formation of ketone bodies and can thus contribute to the ketogenesis in the liver [21,215].

4.4. Excretion

As discussed above, many of the short-chain fatty acids are converted in the hepatocytes into precursor molecules of the gluconeogenesis, aerobic respiration and ketogenesis and thereby obtain a new function in the energy metabolism of the body. However, in the peripheral tissues and the colonocytes short-chain fatty acids are oxidised to generate energy. Here, the short-chain fatty acids are subject to multiple oxidation reactions to form the metabolic end-product CO_2. To date, research on the excretion of the metabolic end-products of the oxidation by these tissues is not available. Possibly, respiratory excretion CO_2 takes place, involving blood for the transport to the lungs. It is not to be ruled out that a fraction of the CO_2 together with water molecules is converted to the weak carbonic acid (H_2CO_3) by potentially present carbonic anhydrase. The corresponding conjugate base (HCO_3^-) might be cleared by the kidneys.

5. Effect of short-chain fatty acids on the metabolism

Short-chain fatty acids appear to have a regulatory function in the cell metabolism of fatty acids, glucose and cholesterol. Recently, it has been discovered that the short-chain fatty acids can act as ligands for a variety of receptors that mediate these regulatory effects. Besides, short-chain fatty acids are found to be important for the regulation of immune responses by the immune system in the microbial environment of the large intestine [28–30]. The effects expressed by short-chain fatty acids on healthy cardiovascular tissue and cardiovascular diseases are largely due to modulation of the metabolism of lipids and glucose. This section discusses applicable receptors and how these regulatory effects are mediated.

5.1. Receptors

The identified receptors, wherein short-chain fatty acids act as a ligand, belong to the G protein-coupled receptor (GPCR) gene family. GPCRs form one of the biggest gene families that could be identified in the human genome [216]. To date, it was possible to characterise approximately 160 receptors from this gene family, which are synthesised from 125 genes. The receptors of which the function and substrates have not been described as of yet, are called *orphan* GPCRs [217].

Recently, it was possible to chart a subset of these orphan GPCRs. The GPR40 subfamily appears to have four members, whose coding genes are all located on chromosome 19q13.1 locus [217]. They appear to feature at least 30% similarity in amino acid sequences [218]. The receptors were discovered with the aid of cosmids (hybrid plasmids with a cos-sequence from the bacteriophage lambda) containing human chromosomal DNA from this locus [217,219]. The four human genes that encode these receptors do not contain introns, which implies that no alternative splicing is possible and that no splice variants of these receptors exist [217]. Studies with BLAST searches of the DNA and protein sequences revealed that this receptor subfamily shows a strong resemblance to the protease-activated receptor GPCR subfamily. However, the long N-termini, which serve as substrates for the proteases for activation and that are characteristic of these receptors, are absent in the four members of the GPR40 subfamily. Therefore, it is plausible that these receptors, GPR40 to GPR43, are activated by a different mechanism [218]. Using ligand fishing strategy, wherein the orphan GPCRs are expressed in yeasts, it was shown that GPR41 and -43 were activated by short-chain carboxylates in a specific an dose-dependent manner [218,220]. Structure-activity studies also revealed that the carboxyl group of the short-chain fatty acids is required for the activation of these receptors. Aldehydes, ketones, alcohols and esters of fatty acids were found to be totally ineffective in this respect. The activation of the GPCRs requires that the carboxyl group of the short-chain fatty acids is positioned at the end of the aliphatic chain. Furthermore, this chain must be composed of no more than six carbon atoms [217]. The optimum chain length for the activation of GPR43 appears to span two to three carbon atoms (acetate and propionate) and ranges from three to five atoms (propionate and butyrate) in the case of GPR41. Short-chain fatty acids featuring deviating lengths up to six carbon atoms are also able to activate these receptors, however activation occurs to a lesser extent [217,218]. The presence of two or more carboxyl groups in the molecule causes the activity to diminish, regardless of the chain length, as was found from studies using various carboxylates including oxalate, malonate, succinate, glutamate and citrate [217].

After the discovery of these receptors, GPR43 was renamed to Ffar2 (free fatty acid receptor 2) and GPR41 to Ffar3. Research using a cell line in which GPR40 was expressed indicated that this receptor does not respond to the administration of short-chain fatty acids [217]. Other studies revealed that GPR40 can be activated by medium-chain fatty acids [221–223]. GPR42 presumably concerns a tandem exon duplication of GPR41 and has lost its ability to be activated by short-chain fatty acids due to mutations. GPR42 uses the promoter of the GPR41 gene and is therefore an open reading frame pseudogene [223]. The gene encoding this receptor differs in six amino acids from the gene encoding GPR41, presumably as a result of mutations which occurred during the gene duplication. Hence, these two receptors of the GPR40 subfamily exhibit about 98% similarity and feature identical amino acid chain lengths [217,219]. The modification of an arginine residue of GPR41 into tryptophan within GPR42 is responsible for the insensitivity to short-chain fatty acids, according to research with rat orthologues. Modification of this amino acid change with the aid of mutagenesis could reduce the activation of rat GPR41 by short-chain fatty acids and partially restore the function of GPR42. Yet it can not be ruled out that GPR42 can be activated by other, unexplored agents. However, this is very unlikely, since GPR42 differs only by a few amino acids residues from GPR41 [218]. Additionally, GPR42 occurs only in a limited portion of the human population, where it is present as a polymorphic insertion in the GPR41 gene [219].

Also, research has been conducted into the intracellular signalling pathways that are activated by ligand binding to Ffar2 (GPR43) and -3 (GPR41). It was found that the intracellular routes of both receptors could be inhibited with pertussis toxins, which implies that the intracellular sides of the receptors are coupled to $G_{i/o}$ proteins. In addition to $G_{i/o}$ proteins, Ffar2 appears to be coupled to G_q proteins in a similar way. Activation of the intracellular signal transduction pathways by the receptors led in both G-protein families to an increase in inositol 1,4,5-triphosphate (IP_3) synthesis, an increase in intracellular calcium concentration, activation of ERK1 and 2 (extracellular signal-regulated kinases; MAPK1 and MAPK3 respectively) and inhibition of the formation of cyclic adenosine monophosphate (cAMP; cyclic AMP) [217].

The short-chain fatty acid receptors also differ with respect to tissue distribution. Various recombinant immune cell models revealed that a strong expression of Ffar2 is observed on leukocytes; particularly on monocytes, B lymphocytes and granulocytes. Also, a lower mRNA expression of this receptor was observed in white and brown adipose tissue, the pancreas, the spleen, bone marrow and the large intestine [217,218,224,225]. Ffar3 has a more comprehensive expression pattern, wherein the highest mRNA expression levels can be detected in the adipose tissue. High mRNA expression levels can also be found in the spleen, pancreas, lymph nodes, bone marrow and to a lesser extent, in granulocytes, cardiomyocytes and vasculature [217,218,225–227].

More recently, it was discovered in a ligand screening test for orphan GPCRs that the murine olfactory receptor 78 (Olfr78) as well as its human orthologue hOR51E2 could be activated by stimulation with short-chain fatty acids [227,228]. However, in contrast to Ffar2 and -3, the receptor responds only to acetate and propionate, but not to butyrate. The Olfr78 receptor appears to be more sensitive to propionate than to acetate with EC_{50} values of 920 μM and 2.35 mM respectively [227]. However, Ffar2 and -3 are significantly more sensitive to these fatty acids with lower EC_{50} values for propionate (259 μM and 274 μM, respectively) and acetate (537 μM and 1.3 mM, respectively) [217,218]. Staining studies have revealed that Olfr78 and its human orthologue hOR51E2 are expressed on some subsets of vascular smooth muscle cells as well as on the renal afferent arteriole [227,229,230].

The strong expression of Ffar2 on various leukocyte populations suggests that short-chain fatty acids are capable of influencing the immune responses of these immune cells [231–234]. This assumption is further supported by the finding of a higher mRNA expression of the receptor during haematopoiesis (differentiation of the leukocytes) and the activation of these cells [218,219]. The same phenomenon could be observed with leukocyte populations in mice, wherein the expression of the murine variant of the GPR43 receptor, LSSIG, also was found to be induced [235]. Short-chain fatty acids exert several effects on the immune cells. Changes in cytoplasmic pH value, an increase in intracellular calcium concentration, increased oxygen consumption, phagocytosis, an increased rate of cell proliferation, increased actin distribution for the formation of the cytoskeleton, an increased granulocyte motility and an enhanced level of chemotaxis could be observed in a study in which short-chain fatty acids were administered to leukocytes [231,236–238]. Short-chain fatty acids are thus capable

of inducing a large number of immunological functions of leukocytes, with the exception of the cell adhesion [239]. The inducing effects are likely to be caused through several mechanisms, wherein at least one signal transduction pathway is activated by the G-proteins that are linked to Ffar2 [236,237].

The physiologically relevant site for the immunoregulatory effect of the short-chain fatty acids is the gastrointestinal tract. Firstly, because of the presence of the enteric microbiota, which makes this environment highly immunogenic and able to exert great influence on the immune system. As a result, there is a great need for regulation of the immune cells in the large intestine [28–30]. Secondly, because the intestinal microflora synthesizes the short-chain fatty acids in sufficiently high concentrations to be able to regulate the leukocytes in this organ effectively [234]. Short-chain fatty acids are formed in the distal regions of the intestinal tract, where the concentrations of the individual fatty acids may rise from 70 to 100 mM and are sufficient to cause the immunoregulatory effects [110]. These effects will occur less in other organs and tissues, as the short-chain fatty acids are present at much lower concentrations in the systemic circulation. As discussed previously, the short-chain fatty acids are preferred for the production of energy in the colonocytes of the intestinal wall and the liver is capable of absorbing a large portion of the short-chain fatty acids from the portal vein. Therefore the fatty acids have a large first pass effect. Accordingly, the concentrations of acetate, propionate and butyrate in the peripheral blood amounts to 100–150, 4–5 and 1–3 μM respectively and thus are likely to be too low to regulate the immune system in the periphery on a large scale [217,240]. However, the concentration of acetate is found to be able to increase tenfold when alcohol is consumed and could potentially reach effective concentrations for the regulatory action on the leukocytes [241,242]. However, it is unclear whether this increase in acetate concentration is present sufficiently long enough in the systemic circulation to be able to observe these effects. After all, acetate can quickly be absorbed by the tissues surrounding the blood vessels, after which it is converted to acetyl-CoA [218].

5.2. Lipid metabolism

Short-chain fatty acids are capable of inhibiting the fatty acid synthesis and lipolysis and can stimulate the oxidation of fatty acids and thermogenesis in the body. Furthermore, short-chain fatty acids cause an increase in mitochondrial function by stimulating the biogenesis of this organelle which, in conjunction with the three previously mentioned effects, leads to induction of the oxidative phosphorylation of short-chain fatty acids through β-oxidation in adipose tissue, the liver and muscles. Therefore, short-chain fatty acids provide a transition from the production of energy-bearing molecules to the consumption of these molecules in these tissues, causing the plasma concentrations of fatty acids to decrease [243–245].

The regulatory effects of short-chain fatty acids on the metabolism of these tissues are established with the aid of the Ffar2 (GPR43) and Ffar3 (GPR41) receptors present on the involved tissues [243]. Moreover, the fatty acids appear to contribute to the activation of 5′-AMP-activated protein kinase (AMPK) in the liver and muscle tissue. The activation of AMPK allows for an increased expression of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), a regulator of the transcriptional activity of different transcription factors that are important for the energy metabolism of cells, and can contribute to the effects of the short-chain fatty acids on the stimulation of the catabolism [246].

AMPK is a heterotrimer, wherein each subunit of AMPK is synthesised from two or more genes. Of the α, β and γ subunits two, two and three isoforms are known respectively, resulting in a total of twelve different AMPK heterotrimers [247]. AMPK is activated by an increase in intracellular adenosine monophosphate (AMP) concentration, as a result of an increased consumption of ATP. In the case of cellular ATP depletion, the enzyme adenylate kinase catalyses the formation of ATP and AMP from two ADP molecules, thereby establishing an increase in the AMP:ATP ratio. This ratio is thus a measure for the cellular energy status and research indicates that AMPK is sensitive to changes in the ratio through four cystathionine beta synthase (CBS) domains in the γ subunit of the protein [248]. For example, ATP appears to be able to inhibit the activation, whereas binding of AMP promotes the activation and prevents deactivation by phosphatases [249–251]. Therefore, stimuli for the activation of AMPK are processes and substances that can inhibit the production of ATP or increase the consumption of this molecule, thereby increasing the AMP:ATP ratio [247,249,252–255].

AMPK has different isoforms of which all show differences in tissue distribution and sensitivity to shifts in the AMP:ATP-ratio [256,257]. Study of the activation of one of the AMPK isoforms, $\alpha_2\beta_2\gamma_1$ AMPK, revealed that the protein moves to the cell nucleus, after AMP has bound to the CBS domain of the γ subunit [248,258]. Subsequently, activation occurs by phosphorylation of the threonine amino acid residue at position 172 in the kinase domain of the protein, which is situated in the α_2 subunit, by AMPK kinase [258–260]. Also increases in AMP concentration and subsequent AMPK activation can also occur without a reduction in ATP, as can be observed when under osmotic stress and when treated with metformin. In these cases, the amount of cellular adenine nucleotides has not decreased, but nonetheless phosphorylation of the threonine residue at position 172 can be observed. However, the associated mechanism of action is not yet known [261–263].

Stimulation of AMPK results in an inhibition of several anabolic processes, such as the fatty acid and cholesterol synthesis and the gluconeogenesis [252–255]. AMPK causes these effects in two different ways. Firstly, AMPK can affect the activity of the enzymes involved in the metabolic reactions by phosphorylation. Secondly, AMPK is able to influence the gene expression of various metabolic enzymes [252].

The activities of the enzymes 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA), glycerol-3-phosphate acyltransferase (GPAT) and acetyl-CoA carboxylase (ACC), which catalyse the initial steps in the pathways of the cholesterol, glycerolipid and fatty acids synthesis respectively, are regulated through dephosphorylation and phosphorylation by the AMP-regulated AMPK [264–268]. Research conducted by Yeh *et al.* showed that inactivation of ACC could be stimulated by AMP and the researchers postulated the existence of an underlying mechanism that inhibits the fatty acid synthesis in the case of a low cellular energy balance [269]. ACC catalyzes the conversion of acetyl-CoA to malonyl-CoA, a precursor molecule of the fatty acid synthesis and a strong inhibitor of carnitine palmitoyltransferase I (CPT-1), a mitochondrial enzyme which is responsible for the absorption of long-chain fatty acids by this organelle [270]. GPAT catalyses the initial conversion in the formation of triacylglycerols by converting glycerol-3-phosphate into lysophosphatidic acid (LPA) [271]. AMPK inhibits these molecules and the associated metabolic pathways by phosphorylating GPAT, HMG-CoA and ACC. The ACC enzyme has two isoforms, ACC-1 and -2, that both can be inhibited by AMPK by phosphorylating three serine residues. In the case of ACC-1 inhibition takes place by phosphorylating the residues at positions 79, 1200 and 1215. The first side of phosphorylation is situated in the N-terminus, whereas the remaining two phosphorylation sites are located between the biotin domain and C-terminus of the enzyme. It is suspected that this terminus of the enzyme mediates the carboxyl transferase activity [264]. The second isoform (ACC-2) was detected and identified with the help

of complementary cDNA [272,273]. ACC-2 shows a strong resemblance to ACC-1 and mainly differs due to an addition of approximately 150 amino acids at the N-terminus. Additionally, expression of ACC-2 appears to a significant degree to be limited to muscle tissue, cardiomyocytes and the liver, while ACC-1 has a broader extent of distribution throughout the body [272–274]. ACC-2 appears able to be inactivated by phosphorylation by AMPK as well. The serine residue, together with the surrounding residues which take care of the orientation of AMPK, is preserved in ACC-2, but has been shifted to position 219 due to the extended N-terminus. The other two phosphorylation sites were found not to have been retained in the second isoform [264,275]. In addition to the inhibition of ACC, GPAT and HMG-CoA, AMPK activates the enzyme malonyl-CoA decarboxylase (MCD) via phosphorylation, which takes care of the degradation of malonyl-CoA in cells [266]. Recapitulating, it can be stated that the inhibition of ACC by AMPK results in a reduced malonyl-CoA production, causing less CPT-1 to be inhibited and therefore increasing the absorption of long chain fatty acids by the mitochondria for β-oxidation in this organelle. This effect is further enhanced by the activation of MCD by AMPK resulting in reductions in the production rate and intracellular concentrations of malonyl-CoA, which thereby causes less inhibition of CPT-1 as well [266]. This implies that the phosphorylations that are carried out by activated AMPK result in a reduced fatty acid synthesis in the liver and consumption of free fatty acids for the formation of other substances, therefore causing larger quantities of these fatty acids to be available for the β-oxidation in the mitochondria of the muscles and liver. These effects are further reinforced by the inhibition of malonyl-CoA production and a decrease in the intracellular malonyl-CoA concentration, causing CPT-1 to be less inhibited by this coenzyme A derivative and promoting the absorption of long chain fatty acids by mitochondria [249,264,276–279].

Apart from direct effects via phosphorylation of metabolic enzymes, AMPK can also exert effects on the gene expression of these enzymes and other proteins [264]. A lot of knowledge of downstream targets of AMPK which can influence the gene expression was gained from research with the AMP analogue 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside 5-aminoimidazole-4-carboxamide riboside (AICAR). AICAR is absorbed by the cells via nucleoside transporters present on the cell membrane. Then AICAR is phosphorylated to the corresponding monophosphate: 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate (ZMP). ZMP was found to have similar effects as AMP on the AMPK system and thus offered the opportunity to study the effects of AMPK following activation [280]. Research using ZMP revealed that induction of AMPK results in the activation of peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) by phosphorylation of a threonine residue at position 177 and a serine residue at position 538. These phosphorylations of PGC-1α enable the co-activator to bind and activate several transcription factors, and thereby influence the expression patterns of genes [246]. In addition, AMPK has proved to be able to inhibit the expression of sterol regulatory element-binding protein (SREBP)-1C. This transcription factor regulates the expression of a number of enzymes that are involved in the synthesis of triglycerides and fatty acids, including ACC, GPAT and fatty acid synthase (FAS). Inhibition of this transcription factor causes a decrease in the intracellular concentration of these enzymes, which result in a reduced production of triacylglycerides by esterification and a further enhanced oxidation of the fatty acids [260,266,281,282].

PGC-1α is the first member of the PGC-1 family of co-activators and was identified as a PPARγ-interacting protein from the brown adipose tissue [283]. This co-activator appears to be particularly involved in the regulation of the energy metabolism of cells by interacting with transcription factors, estrogen-related receptor alpha, nuclear respiratory factor (NRF) 1 and -2, peroxisome proliferator-activated receptors (PPAR) α, -β and -γ, liver X receptor (LXR) and the farnesoid X receptor. All of these transcription factors are able to influence the regulation of the cholesterol, lipid and glucose metabolism in cells [245,246]. The co-activation of NRF-1 and -2 by PGC-1α leads to an increased expression of mitochondrial transcription factor A (TFAM), a mitochondrial matrix protein that is important for the replication and transcription of mitochondrial DNA material. TFAM promotes the replication of the genetic material required for mitochondrial biogenesis in both the nuclear and mitochondrial genome. The induction of the mitochondrial biogenesis by PGC-1α results in an increased cellular enzymatic capacity for the β-oxidation, the Krebs cycle and the oxidative phosphorylation [245,284–288].

PGC-1α also proves to be able to promote the expression and activation of PPARα. PPARα regulates the expression of a large number of fatty acid-oxidizing enzymes, such as acyl-CoA oxidase (ACO [289]), carnitine palmitoyltransferase-1 (CPT-1 [290]) and uncoupling protein 2 (UCP2 [291]). The upregulation of the synthesis of these enzymes results in an enhanced enzymatic capacity for the mitochondrial oxidation of fatty acids. Furthermore, PGC-1α affects the adaptive thermogenesis in the brown adipose, by the increased expression of both UCP1 and -2. Both proteins stimulate thermogenesis in this tissue and induce the breakdown of fatty acids for the generation of heat [245,283]. This feature is further confirmed by the finding that PGC-1α is strongly induced in this tissue when it is exposed to low temperatures [245].

The co-activators of the PGC-1 gene family do not have any histone acetyltransferase (HAT) activity in their primary amino acid sequence and are therefore dependent on other proteins, mainly other co-activators, which are capable of modifying the chromatin structure of the DNA, in order to be able to bind, together with a transcription factor, to the genetic material and to effectively influence its expression. For this purpose, the PGC-1 co-activators bind HAT activity-containing proteins at their N-termini, such as CREB-binding protein (CBP), p300 and steroid receptor co-activator-1 (SRC-1) [292]. These proteins are capable of unwinding the DNA by applying acetylations on the histones, after which the PGC-1α bound transcription factors can influence the expression of various genes [293,294].

In addition to the induction of the expression of other genes, PGC-1α also appears to influence its own formation. Research wherein AICAR was administered to cells together with small interfering RNA (siRNA) for switching off the expression of PGC-1α, demonstrated that the long-lasting increase in the fatty acid oxidation remained absent [295]. From the results of the study two conclusions could be drawn. First of all, for the long-term increase in the mitochondrial fatty acid oxidation, AMPK appears to be dependent on the modulation of the gene expression of PGC-1α. PGC-1α induction occurs through phosphorylation by AMPK, which enables PGC-1α to bind its own 2 kb promoter and induce its gene expression. This finding is further confirmed by research with AMP analogue AICAR, which demonstrated that the inducing ability of PGC-1α disappeared by mutation of the two phosphorylation sites of AMPK in this protein [246,295]. Secondly, it appears that the increase in fatty acid oxidation by PGC-1α is dependent on the induction of its own expression. Application of siRNA for PGC-1α also causes elimination of the stimulatory effects of this co-activator on the fatty acid oxidation. This indicates that PGC-1α activation through phosphorylation by AMPK results in a feed-forward loop of increased PGC-1α expression, prior to stimulation of the fatty acid oxidation by this protein [246,296].

Another mechanism of the cell which can stimulate fatty acid oxidation is the prevention of degradation of PGC-1α, which

prolongs its effectiveness. To this end, the mitogen-activated protein kinase (MAPK) p38 is activated by AMPK, resulting in an increase of the relatively short half-life of PGC-1α of approximately 2.3 h. For this increase of the half-life, the MAP kinase phosphorylates three amino acid residues; two threonine residues at sites 262 and 298 as well as a serine residue at position 265. An additional effect of these phosphorylations which further stimulates the effects of PGC-1α, is the displacement of the binding site of the p160 myb-binding protein (p160MBP). p160MBP is a potent transcription inhibitor that normally inhibits the functions of PGC-1α by binding to its central domain. Relocation of the binding site outside of the central domain suppresses its inhibition, causing PGC-1α to become more effective [245,297–300].

Short-chain fatty acids prove to have an effect on the above-described fatty acid oxidation stimulating processes. A study was conducted were high-fat meals were administered to two groups of mice, wherein one group five weight percent of butyrate was added to the meal. It was found that butyrate was able to raise the mitochondrial respiration. In comparison to the control group, an increase in the consumption of oxygen and an increased production and release of carbon dioxide could be measured after the administration of the butyrate-containing meals. Additionally, it was discovered that an increased expression of PGC-1α, PPARδ and CPT-1b in cells of the brown adipose tissue and the muscle can contribute to enhancement of the mitochondrial function. The plasma concentrations of triglycerides, cholesterol and fatty acids were found to decrease with an increase of the butyrate concentration in the plasma. It appears that short-chain fatty acids are able to activate AMPK [244]. As it turned out, butyrate is able to activate this kinase in cells of the Caco-2 cell line [301]. Also, butyrate seems to be able to activate PGC-1α and to regulate the expression of the co-activator. The mice that received the butyrate-containing nutrition showed a strong increase in activated PGC-1α protein and PGC-1α mRNA in the brown adipose tissue [302,303]. The enhanced expression of PGC-1α and the other transcription factors may be the result of the ability of butyrate to inhibit histone deacetylases class 1 and 2, resulting in an increased acetylation of histones, which allows a potentially stronger expression of genes. This modification of the chromatin structure is possibly present in the promoter regions of the genes encoding PGC-1α, PPARδ and CPT-1b, which would allow upregulation. The inhibition of histone deacetylases seems restricted to butyrate, which can achieve this effect at low concentrations. Other research showed that a relatively high plasma concentration of 0.2 mM acetate was not sufficient to prevent the deacetylation of histones [304–306]. Despite the fact that direct activation of AMPK by short-chain fatty acids could be observed, the underlying mechanism remains to be determined. The activation of AMPK by short-chain fatty acids and the inhibition of histone deacetylases by butyrate could contribute to the upregulation of PGC-1α [244].

In addition to the direct activation of AMPK, short-chain fatty acids also prove to be able to activate this kinase by altering the AMP:ATP-ratio in the liver. An *in vitro* study revealed that butyrate is able to increase both the formation of AMP as well as the ATP consumption in this organ. However, the mechanisms involved in the stimulation of the consumption of ATP and AMP formation in the liver by butyrate are still unknown [244]. A similar effect was observed in a study in which acetate was administered to mice. A large portion of the administered acetate was rapidly absorbed by the liver and metabolised to acetyl-CoA by acetyl-coenzyme A synthetase (ACS), resulting in the formation of AMP at the expense of intracellular ATP [307,308]. The overall reaction catalysed by ACS is shown below.

$$\text{ATP} + \text{acetate} + \text{co-enzymeA} \leftrightarrow \text{AMP} + \text{pyrophosphate} + \text{acetyl-CoA}$$

Reaction catalysed by acetyl-coenzyme A synthetase [307].

The increase in the AMP:ATP-ratio by administration of the short-chain fatty acids can then induce the phosphorylation of AMPK, causing this kinase to become activated [309–312].

A third way in which short-chain fatty acids can contribute to the oxidation of fatty acids is via stimulation of the Ffar2 (GPR43) and Ffar3 (GPR41), which are expressed in the white adipose tissue. Adipocytes store triglycerides in case of excessive energy intake and release these lipids when there is a need for more energy [313]. Apart from regulating the storage and release of energy, the white adipose tissue also is a major production site for endocrine factors [314]. Stimulation of these receptors by the fatty acids results in a reduced lipolysis, an increased production and secretion of leptin and a decreased fat storage in this tissue [243,315–317].

In case of energy shortage, the triglycerides stored in the white adipose tissue are cleaved to free fatty acids by means of hydrolysis of the ester bond, after which these fatty acids are released into the bloodstream to be oxidised elsewhere in the body for the release of energy. This process is called lipolysis and is regulated by means of various hormone receptors, which are present on the cell membranes of the adipocytes. The primary inducing lipolysis system of the reactions uses the intracellular cAMP pathway. Several hormone receptors and β-adrenergic receptors are intracellularly connected to the G_s-α subunit of the G protein family, which is capable of activating the signal transduction pathway after dissociation of the heterotrimer. The free $_{\alpha s}$ subunit stimulates adenylate cyclase (AC), which forms cyclic AMP (cAMP) from ATP. As a result of the increased intracellular concentration of cAMP, the cAMP-dependent protein kinase A (PKA) becomes activated. The now active PKA can then phosphorylate and stimulate the hormone sensitive lipase (HSL) enzyme. Subsequently, HSL hydrolyses the ester bonds in tri- and diglycerides in the perilipins of the adipocytes in the white adipose tissue to free fatty acids, which are then secreted into the blood [318–322].

The activity of HSL is regulated by two serine residues that can be phosphorylated by PKA [323,324]. The serine residue at position 563 is phosphorylated by activated PKA and stimulates the lipolysis activity of the enzyme [323]. The second residue at position 565 acts as a regulator for the phosphorylation of the residue at position 563 and is present in phosphorylated form in the adipocytes at a basal level [324]. The phosphorylation of this residue inhibits the phosphorylation of the serine residue at position 563, resulting in a reduction of the lipase activity of HSL. Apart from PKA, other kinases, such as glycogen synthase kinase (GSK) and AMPK, are also able to phosphorylate this second amino acid residue [325].

Several *in vivo* studies have shown that short-chain fatty acids are able to cause a reduced lipolysis and decreased fatty acid concentration in the blood plasma via activation of the short-chain fatty acid receptors, Ffar2 and -3, in the white adipose tissue [243,316,317]. As mentioned earlier, the receptors at the intracellular side are coupled to members of the $G_{i/o}$ protein family [217]. Research using pertussis toxin and a β3-receptor agonist revealed that inhibition of G_i proteins resulted in a stronger stimulation of the lipolytic activity in adipocytes than the administration of the agonist only, indicating that G_i proteins are involved in the regulation of the lipolytic activity in white adipose tissue [326]. Activation of the receptors is responsible for the release of the G_i-α subunit, which has an inhibitory effect on adenylate cyclase. Inhibition of AC causes a reduced conversion of ATP to cAMP, causing the cAMP-dependent PKA to become less active and no longer able to phosphorylate HSL. Consequently, HSL is present in less active form, therefore leading to a reduced lypolytic activity of the enzyme and less hydrolysis of triglycerides. This is accompanied by a reduction in the synthesis and release of free fatty acids

into the blood, causing the fatty acid concentration in the blood plasma to decrease [327].

The adipocytes of the white adipose tissue also produce leptin, after which this protein is secreted into the blood [313]. Studies with wild-type and leptin-deficient ob/ob mice showed that exogenous administration of leptin causes a reduced food intake, a decrease in body weight and an increase in energy consumption in both types of mice [328,329]. Therefore, leptin is a potent anorexigenic hormone that appears to be able to suppress food intake using receptors of the central nervous system. The plasma concentration of the hormone shows a positive correlation with the adiposity and can be used as a measure of the amount of stored body fat. Fasting causes a decrease in the leptin concentration, while excessive food intake causes it to increase [330–333]. From this it seems that leptin prevents the accumulation of lipids in the adipose tissue by restricting the food intake. In addition, the hormone seems to be able to suppress the triglyceride synthesis and stimulate fatty acid oxidation via the activation of AMPK [266,334]. As a matter of fact, leptin proves to be able to stimulate AMP synthesis, causing the AMP:ATP-ratio in the liver to increase, which allows activation of AMPK in this organ. Additionally, the hormone shows to be able to directly stimulate the phosphorylation of the α_2 catalytic domain of AMPK [266,335]. However, the mechanisms underlying the induction of AMPK by leptin are still unknown.

Recently, it has been discovered that Ffar2 and -3 both can stimulate the release of leptin through the white adipose tissue [315]. Propionate as well as butyrate were found to stimulate the secretion of leptin by the adipose tissue, causing the plasma concentration of leptin to increase. Using studies with mice in which sodium propionate was administered, it was possible to detect an increase in the concentration of leptin in the blood plasma approximately seven hours after administration. *In vitro* studies in which cells of the adipocyte Ob-Luc cell line and cultured mouse adipocytes were pre-treated with pertussis toxins, showed no increase in the secretion of leptin after administration of propionate. This demonstrates that the pre-treatment, wherein G_i proteins are inhibited, causes the stimulatory effect of the short-chain fatty acids to disappear and that the induction of the secretion by the fatty acids is dependent on intracellular G_i proteins linked to the Ffar2 and -3 receptors on the adipocytes [332]. Also, activated PGC-1α can cause an enhanced gene expression of leptin, since the gene promoter of the hormone is found to be sensitive to stimulation by the transcription factor PPARγ, which can be activated by the PGC-1α. Activation of AMPK by short-chain fatty acids or by the increased plasma concentration of leptin may thus lead to an enhanced production of leptin in the white adipose tissue [336–340].

Besides the aforementioned effects, Ffar2 also appears to affect the absorption and storage of lipids in the white adipose tissue. Insulin causes a stimulation of the absorption of fatty acids in this tissue by binding to the insulin receptors on the cell membranes of the adipocytes. The binding leads to intracellular activation of phosphoinositide 3-kinase (PI3K), which catalyses the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol (3,4,5)-trisphosphate (PIP3) [341]. The increase in the intracellular PIP3 concentration leads to activation of Akt (protein kinase B; PKB), that regulates the storage of fats [342]. Stimulation of Ffar2 by extracellular binding of short-chain fatty acids causes the intracellular, heterotrimeric $G_{i/o}$ protein to dissociate. This dissociation leads to subsequent activation of phospholipase C (PLC) and protein kinase C (PKC). PKC then activates the phosphatase and tensin homolog (PTEN), a phosphatase that catalyses the reaction of PIP3 to PIP2, by means phosphorylation. As the intracellular concentration of PIP3 decreases by the activation of PTEN, Akt becomes inactive and the insulin-activated Akt signal transduction is inhibited. As a result of this inhibition, the insulin sensitivity decreases and the accumulation of fat in the adipocytes of the white adipose tissue is prevented. Research using a glucose clamp test provided that decreased sensitivity to insulin, leads to an increased sensitivity for this protein in other tissues. The consumption of both glucose and lipids for the generation of energy were shown to increase in both the liver and muscles. In this way Ffar2 is able to stimulate the consumption of lipids in other tissues, whilst inhibiting the storage of fat in white adipose tissue [243].

5.3. Glucose metabolism

In addition to the regulation of the metabolism of lipids, short-chain fatty acids can also affect the metabolism of glucose. Several studies with various animal and cell models revealed that the fatty acids can reduce the glucose concentration in the blood. The observed reductions are caused by several mechanisms which are mediated by short-chain fatty acid receptors Ffar2 and -3 and AMPK. In addition, some of the effects are caused by the incretins glucagon-like peptide-1 (GLP-1) and peptide YY (PYY).

Research with rats and diabetic hyperglycaemic KK-A(y) mice has shown that oral administration of propionate or acetate both lead to a decrease of the glucose content in the blood [309,343,344]. This effect can most likely be attributed to the activation of hepatic AMPK due to the influence of the administered short-chain fatty acids on the AMP:ATP-ratio [244]. As mentioned previously, AMPK regulates the gene expression of various metabolic enzymes and appears to be able to inhibit the hepatic gluconeogenesis [309–311,345]. Activated AMPK is able of effectively inhibiting two of the enzymes, glucose-6-phosphatase (G6Pase) and phopshoenolpyruvate carboxykinase (PEPCK), involved in the synthesis of glucose in the liver. Quantitative analysis of the mRNA of these enzymes from the cytoplasm of the hepatocytes yields that treatment with acetate and propionate leads to a reduced expression of both PEPCK and G6Pase [309–311,346]. In the case of induction of G6Pase with dexamethasone, acetate proved to be able to effectively inhibit the mRNA expression of the enzyme [309]. The decrease in the glucose concentration in the blood plasma therefore seems to be primarily caused by inhibiting gluconeogenesis in the liver [309–311].

Another effect that can contribute to the decrease in the plasma glucose level in the blood is the stimulation of fatty acid oxidation in the liver, muscle and the brown adipose tissue and inhibition of lipolysis in the white adipose tissue by short-chain fatty acids. High plasma concentrations of fatty acids can reduce the sensitivity to insulin of different tissues and hinder the absorption of glucose. Stimulation of the oxidation of fatty acids in the three previously mentioned tissues causes a decrease in the free fatty acid concentration in the blood plasma, which leads to an increase in the sensitivity to insulin and thus to an enhanced absorption of glucose [347–351]. As a result of the stimulation of the oxidation of fatty acids by the short-chain fatty acids, the glucose absorbed by the tissues is converted to glycogen [352,353]. In a study in which rats were administered propionate a decrease in the maximum insulin secretion and glucose tolerance area under the curve could be observed with the aid of an insulin increment test, which indicates an increase in the insulin sensitivity [343]. This phenomenon has further been confirmed by studies in which hyperglycaemic KK-A(y) mice were administered acetate. After eight weeks, an increase in the glycogen storage in the liver and muscle tissue by a factor of 1.9 and 10, respectively, could be detected when compared with the control group [309]. A stimulation of the storage of glycogen could also be observed in a study in which AMPK was activated by long-term administration of AMP analogue AICAR [352,353]. The

improvement in insulin sensitivity by stimulation of fatty acid oxidation by the short-chain fatty acids may lead to a further decrease in the glucose concentration [354,355].

In order to prevent damage to the body resulting from an unhealthy diet, for example in the case of eating disorders, the body features a complex neuro-endocrine system, which enables signal transduction of the energetic status of the peripheral tissues to the central nervous system. The central nervous system can then regulate food intake and energy consumption of the body in order to pursue an appropriate energy balance [356]. L cells are part of the enteroendocrine system and span across the entire length of the intestinal epithelium. These cells enable the release of incretins GLP-1 and PYY, which can influence the glucose metabolism [357]. The release of these incretins by these cells appears to be affected by short-chain fatty acids, which activate the fatty acid receptors Ffar2 and -3 that are present on the apical membrane of the enteroendocytes. Research with Ffar2 and -3 knock-out mice showed a significantly reduced release of both GLP-1 and PYY and emphasises the importance of these receptors for the release of these hormones [358]. With the aid of immunohistochemistry and colocalization studies it was possible to demonstrate the expression of Ffar2 on PYY-containing enteroendocytes [359,360]. Additionally, a dot pattern could be observed on the GPR43 immunoreactive enteroendocytes, which may be caused by the presence of Ffar2 receptors in the Golgi apparatus for transport to the apical membrane of the endocyte [359]. Using quantitative reverse transcription polymerase chain reaction (RT-PCR) it could be determined that Ffar2 mRNA was expressed most strongly in the mucosa and intestinal wall of the more distal parts of the gastrointestinal tract, such as the terminal portion of the ileum and the colon [359–361]. Immunohistochemical provisions of the expression sites of Ffar3 showed that this receptor was present primarily to the apical side of the cytoplasm of enteroendocytes present in the mucosa, but not on the apical membrane. Immunohistochemical investigation into the expression sites of Ffar3 indicated that this receptor was present primarily on the apical side of the cytoplasm of enteroendocytes present in the mucosa, but not on the apical membrane. Here, the immunoreactivity seemed concentrated around the endoplasmic reticulum and the Golgi apparatus of the PYY-containing enteroendocytes. Possibly, the receptor had yet to be transported and placed on the apical membrane or was internalised at the moment of the determination [362]. When comparing the results of the receptors of these tissues to those of other tissues, the two receptors appear to be less expressed in the gastrointestinal tract than in the spleen or bone marrow [217,218]. There is also a difference in expression between the two receptors in the human colon. Comparison of the results of immunohistochemical studies revealed that all PYY-containing enteroendocytes express Ffar2 on their apical membranes, whereas the expression of Ffar3 is limited to just a fraction of these cells. Therefore, the strongest stimulatory effect on the release of incretins by short-chain fatty acids is most likely caused by activation of the Ffar2 on the enteroendocytes [359,360,362]. Both receptors are intracellularly coupled to $G_{i/o}$ proteins, whereby activation of the receptor leads to an increase of the intracellular calcium concentration and a decrease in cAMP due to inhibition of adenylate cyclase. Both calcium and cAMP act as intracellular second-messenger and promote the fluid secretion in a synergistic manner, thereby stimulating the release of PYY and GLP-1 by enteroendocytes [217,218,363]. The results of various animal and cell culture studies suggest that short-chain fatty acids, in addition to their ability to directly induce the release of the incretins, can also induce the gene expression of PYY and proglucagon (GCG), the gene encoding GLP-1 [364]. An increased synthesis of the hormones can contribute to an even higher release of the incretins, but the way in which the upregulation occurs is unclear to date [365].

Endocrine L cells in the distal parts of the intestinal tract are the major site of production of peptide YY, but PYY is also produced to a lesser extent in the α-cells of the islets of Langerhans, the stomach and the brain stem [366–368]. PYY affects appetite via the control center, the arcuate nucleus, in the hypothalamus, where two different types of neurons control this regulation. The first system in the arcuate nucleus makes use of neuropeptide Y (NPY) neurons, which stimulate the appetite, and thus cause an orexigenic effect. The second system has an opposite, anorexigenic effect, and makes use of neurons that release α-melanocyte-stimulating hormone (α-MSH); one of the cleavage products of pro-opiomelanocortin (POMC) [369]. The peripheral satiety hormones seem well capable to reach and activate the arcuate neurons. A study on the inhibition of appetite by activation of the leptin receptors on NPY neurons in the arcuate neurons, confirms the reachability of this brain region for saturation signals from the periphery [360,370]. Activation of each of the systems in turn leads to signal transduction to other regions in the brain, where the actual regulation of the appetite takes place [34]. In addition, both hypothalamic systems appear to be able to adjust the tissue sensitivity to insulin. Infusion of NPY in the arcuate nucleus of rats led to increased insulin resistance and hyperinsulinaemia [371,372]. However, when α-MSH was administered to this brain region, an improvement in insulin sensitivity could be observed [373]. From this, it can be deducted that hypothalamic NPY over-expression may lead to an increased insulin resistance, which can result in the development of, for example, obesity or type 2 diabetes mellitus [371,372]. In contrast, stimulation of melanocortin receptors 3 and 4 by activation of the α-MSH secreting neurons or administration of this peptide can contribute to an improvement in insulin sensitivity [373].

In the L cells two forms of the hormone, having opposite effects on the regulation of appetite, are produced; PPY_{1-36} and PYY_{3-36} [374]. PYY_{1-36} proves to be able to stimulate the appetite, whereas PYY_{3-36}, the most common form of PYY in the circulation, inhibits the appetite [375–377]. PYY_{1-36} has proved able to bind all the neuropeptide Y receptors with different affinities. The second form of the protein, PYY_{3-36}, turns out to be more selective for the Y2 receptor and to a lesser extent for the Y5 receptor [374,378–381]. The Y2 receptor is found presynaptically on the NPY neurons in the arcuate nucleus and allows for an inhibition of the orexigenic effect [375,382]. Binding of PYY_{3-36} to the Y2 receptor results in inhibition of the NPY neurons and for a stimulation of the appetite inhibition and the insulin sensitivity increasing effect of the α-MSH-secreting neurons in the arcuate nucleus [375]. Studies in which rats were administered PYY_{3-36} peripherally or directly into the arcuate nucleus, showed a strong decrease in appetite and body weight at very low concentrations of the peptide (100 fmol) [375]. Furthermore, research using *in situ* hybridization, in which the mice were administered PYY_{3-36} via an intraperitoneal injection, shows that the incretin causes a downregulation of NPY mRNA and an upregulation of POMC mRNA in the arcuate nucleus [380]. Also, the administration of the peptide resulted in an improved glucose uptake in muscle and fat tissue under hyperinsulinaemic conditions, which enhances the role of PYY_{3-36} in the improvement of insulin sensitivity in these tissues. In the case of hyperinsulinaemia the absorption of glucose by these tissues is highly restricted, implying that more insulin needs to be released from the pancreas in order to compensate for the loss of sensitivity of the muscle and fat tissue [383]. Research indicates that hyperinsulinaemic conditions may stimulate the development of obesity. However, the administration of PYY_{3-36} may potentially contribute to the prevention of this disorder by counteracting the acquired insulin resistance through inhibition of the orexigenic system in the hypothalamus [383–385].

Incretin release by enteroendocytes occurs approximately fifteen minutes after the ingestion of food. The amount of release appears proportional to the caloric content of the food ingested and results in an approximately six-hour increase in the plasma concentrations of PYY [386–389]. The initial release of incretins by the L cells seems to be caused by an indirect neuronal reflex, but the continuous secretion appears to be the result of stimulation of the L cells by the intraluminal contents of the intestine [390].

In addition to regulation of appetite, the release of PYY by L cells also appears to decrease the gut motility. Several studies have shown that short-chain fatty acids can induce the decrease of the contractile activity of the longitudinal muscle of the colon by the release of PYY. This phenomenon is also called the colonic brake or ileal brake and is capable of modulating the transit time of substrates passing through the intestinal tract [365,368,391–395]. The enhanced release of PYY to the portal vein through activation of Ffar2 by the fatty acids allows for activation of many β-adrenergic, serotonergic, and opioid pathways, which decrease the contractile activity in the colon [396,397].

The second incretin that is secreted by the L cells into the portal vein following food intake is GLP-1. GLP-1 is formed by the L cells by intracellular cleavage of the proglucagon precursor protein [357,398,399]. After the incretin is released into the blood, it appears to be able to stimulate glucose-dependent insulin secretion from the β-cells and to inhibit the glucagon secretion by the α-cells in the pancreas. In addition, GLP-1 stimulates the proliferation of β-cells and inhibits the apoptosis of these cells in the pancreas [398,400,401]. All of these effects of GLP-1 on insulin secretion and β-cells are called the incretin effect [357,398]. GLP-1 uses the glucagon-like peptide 1 receptor (GLP1R), which is expressed in the hindbrain and the arcuate nucleus, for achieving these effects [402]. Activation of the GLP1R in arcuate nucleus causes an increase in the sensitivity of the islets of Langerhans for the glucose concentration in the blood plasma, resulting in stimulation of the glucose-dependent insulin secretion by the β-cells [403–406]. The increased release of insulin is achieved via inhibitory $GABA_A$ receptors, which are expressed on the α-cells in islets of Langerhans, that inhibit the glucagon secretion and further reduce the plasma concentration of glucose through inhibition of the conversion of stored glycogen to glucose [407,408]. As indicated earlier, short-chain fatty acids can induce the gene expression of proglucagon in enteroendocytes by activation of the Ffar2 receptors that are present on the apical membrane of these cells. In addition to stimulation of the production, the fatty acids also induce the secretion of the incretins through the Ffar2 receptor, resulting in more GLP-1 that is released into the portal vein [409,410].

5.4. Cholesterol metabolism

Several studies show that the administration of short-chain fatty acids or dietary fibre to both rats and humans causes a decline in the plasma concentrations of cholesterol [264,282,411–416]. Furthermore, a daily administration of short-chain fatty acids during a month resulted in a significant, dose-dependent decrease in body weight in subjects with obesity [412]. Cholesterol has a multitude of important functions for the survival of vertebrate organisms. For instance, the molecule is important for the preservation of the structure of cell membranes and for a variety of cell functions. Additionally, cholesterol is an important precursor for the synthesis of bile acids, steroid hormones and oxysterols, which are all important for the maintenance of the organism [417]. The cholesterol in mammals originates from two sources; cholesterol can be absorbed from the diet, but can also be synthesised from acetyl-CoA by the liver [267,418,419]. Therefore the liver is the major regulatory site of cholesterol homeostasis. This organ facilitates not only the synthesis of cholesterol, but also the breakdown and excretion of cholesterol, bile acids and salts through bile into the faeces as well as the synthesis and release of both very-low-density and high-density lipoproteins (VLDL and HDL, respectively). In this way, the liver is very important for the distribution of cholesterol throughout the body and also strongly contributes to the reverse cholesterol transport (RCT), in which cholesterol is retrieved from the peripheral tissues and transported to the liver [420–422]. RCT mainly uses HDL particles to collect cholesterol from the periphery, which are then absorbed by the hepatocytes via the scavenger receptor class B member 1 (SR-B1) receptors that are expressed on the basolateral membrane of these cells. Subsequently, the cholesterol can be secreted into the bile in unaltered form or can be metabolised by cholesterol 7 α-hydroxylase (CYP7A1) to neutral sterols or bile acids, after which these metabolites as well as cholesterol will be released into the bile and excreted in the faeces [422,423].

Cholesterol is synthesised from acetyl-CoA via the sterol synthesis/mevalonate pathway in the hepatocytes [424,425]. The importance of the cholesterol synthesis from acetyl-CoA by the hepatocytes for the survival is illustrated by the observation that when defects in the cholesterol synthesis pathway were introduced in mice, these defects were lethal. Complete loss of cholesterogenic enzymes hardly occurs and reduced function of these enzymes leads to the development of severe diseases [426].

The mevalonate pathway produces cholesterol using various enzymatic conversions, wherein HMG-CoA reductase is the rate-limiting enzyme in this synthesis pathway [427]. Both a mixture of butyrate, propionate and acetate as the separate short-chain fatty acids appear to be capable of reducing the hepatic cholesterol synthesis rates by inhibiting the enzymes involved in the synthesis of the lipid, such as HMG-CoA reductase and HMG-CoA synthase [424,428,429]. The activity of HMG-CoA is regulated by a reversible phosphorylation of the serine residue at position 871, wherein dephosphorylation results in activation of the enzyme. The serine residue is mainly phosphorylated and inhibited by activated AMPK. Therefore, the ability of short-chain fatty acids to increase the AMP concentration in the liver, most likely causes the observed inhibition of the cholesterol synthesis by the activation of AMPK [268,430–435].

Apart from the decrease in the serum concentration of cholesterol, *in vivo* studies also revealed a decrease in the concentration of hepatic HMG-CoA after the administration of short-chain fatty acids. This reduction is caused by an inhibition of the gene expression of ATP citrate lyase (ATP-CL); an enzyme that is involved in the formation of acetyl-CoA, a precursor molecule for cholesterol, in the cytoplasm of the hepatocytes. The activation of AMPK by the fatty acids results in the inhibition of SREBP-1c, a transcription factor, which regulates the expression of ATP-CL. The inhibition of gene expression leads to lower levels of cytoplasmic ATP-CL and thus to a reduced available amount of acetyl-CoA for the synthesis of cholesterol via the mevalonate pathway [282,288].

Cholesterol is able to stimulate the synthesis of triglycerides in the liver, but the addition of acetate to a cholesterol-rich diet caused this stimulatory effect to disappear and decreased the overall formation of triacylglycerides in this organ. It is postulated that the induction of fatty acid synthesis by the addition of acetate leads to an increased gene expression of alternative oxidase (AOX), which results in a reduced production of triglycerides [282]. In addition to the reduction of the formation of cholesterol, short-chain fatty acids are also able to speed up the excretion of cholesterol by increasing the production of secretin, which causes the hepatocytes to excrete more cholesterol-containing bile [436].

6. Effect of short-chain fatty acids on the cardiovascular system

Research over the past twenty years has demonstrated that the endothelial layer of the cardiovascular system is more than a single layer of cells to cover the muscle tissue of the blood vessels. For example, the endothelium appears to play a major role in the regulation of blood pressure and the free flow of blood. In addition, it prevents bleeding, can inhibit or stimulate inflammatory reactions and can affect the function of nearby vascular muscle tissue in several ways [437].

Disorders of the cardiovascular system are generally caused by disturbances of the functions of the endothelium. The main causes of these disturbances can be traced back to metabolic imbalances and their consequences. Several studies revealed that hyperglycaemia, insulin resistance and dyslipidemia can all cause endothelial dysfunction and low-grade inflammation in this tissue [25–27]. The aforementioned metabolic imbalances can be caused by a variety of disorders, such as obesity, hypertension and type 2 diabetes mellitus and therefore constitute risk factors for the development of cardiovascular diseases [1,25,438]. Various studies have found that diabetic patients are two to four times more likely to die from cardiovascular disease than non-diabetics [439–441].

Loss of endothelial function by these diseases leads to accumulation of lipids and inflammatory cells in the subendothelial space, causing reduction of the inner diameter of the blood vessel. The protraction of this phenomenon leads to atherosclerosis; the clogging and hardening of the arteries. In this process a plaque is built at the inside of a blood vessel, which hinders the blood flow. When the blood vessel is completely obstructed and the downstream tissues are no longer provided with nutrients and oxygen, this will lead in an ischemic attack and may cause necrosis in these tissues. Additionally, rupture of an atherosclerotic plaque can occur, leading to the release of a large amount of coagulation factors which initiate the coagulation cascade. As a result, clogging of blood vessels elsewhere in the body can occur. Common sites for the occurrence of an embolism are the coronary arteries, which supply the heart with oxygen, and the cerebral blood vessels. Furthermore, in the case of hypertriglyceridemia, accumulation of fat in the heart muscle can take place, causing the heart to enlarge and making it less able to contract [438,442–444].

As described in the previous chapter, short-chain fatty acids affect several metabolic processes in the body and therefore appear suitable for the prevention and treatment of endothelial dysfunction and its effects. Besides the effects on metabolic processes, the fatty acids have an anti-inflammatory effect, which allows inhibition of the release of inflammatory mediators and the influx of immune cells to the site of inflammation.

In the rest of this chapter the physiological role of healthy cardiovascular tissue and how different risk factors can disrupt endothelial functions will be discussed. Then, it will be discussed how this loss of function leads to the development of atherosclerosis and how a high lipid concentration in the blood may result in the accumulation of fat in the heart muscle. In the remainder of the chapter, the ways in which short-chain fatty acids can contribute to the prevention and treatment of the loss of endothelial function will be addressed.

6.1. Physiological functions of healthy endothelial tissue

Endothelial tissue appears to have a number of important regulatory functions in ensuring an unhindered blood circulation. For the regulation of the flow of blood to the various tissues and organs, the endothelium releases various chemical mediators. In order to achieve vasodilation, the endothelial cells produce nitric oxide by converting the amino acid L-arginine to L-citrulline by endothelial nitric oxide synthase (eNOS); an enzyme which is present in these cells. The cells produce picomolar concentrations of nitric oxide, which are delivered to adjacent smooth muscle of the blood vessel. In the muscle cells, nitric oxide activates the enzyme guanylate cyclase, which leads to an increase in the intracellular cyclic GMP concentration that causes relaxation of the muscle cells [445]. Vasoconstriction is achieved through the release of endogenous produced endothelin to the muscle cells by the endothelium [446]. Under physiological conditions, there is a balance between the endothelium-derived relaxing and contracting factors, but this balance appears to be shifted in diabetic patients and atherosclerosis in patients, which may lead to loss of the endothelial functions [437,447,448].

Moreover, the endothelium regulates inflammatory reactions and the blood coagulation. Healthy endothelial tissue appears to be able to inhibit the adhesion of platelets and leukocytes to the vascular surface and a balance between the coagulation and fibrinolytic systems is maintained. Apart from a physiological barrier between the blood circulation and the vascular muscle tissue, the endothelium also prevents the development of atherosclerosis in the vascular and thrombus formation in the blood by limiting vascular adhesion [445,449–452].

Finally, the endothelium also has a role in the regulation of the proliferation and differentiation of the vascular muscle tissue. To this end, the endothelium produces a number of mediators, which are then delivered to the adjacent muscle cells via diffusion [451,453–456].

6.2. Metabolic imbalances

As mentioned previously, hyperglycaemia, insulin resistance and dyslipidemia are the main forms of metabolic imbalance that can lead to disturbance of the endothelial functions [25–27]. They frequently occur in patients with obesity or type 2 diabetes mellitus. The different metabolic imbalances lead, together and separately, to the disruption of endothelial functions and thus to an increased risk of the manifestation of cardiovascular disease. First, the ways in which the complex of imbalances can lead to disruption of the endothelium will be addressed, after which the specific effects of individual metabolic imbalances will be discussed.

Insulin resistance is a common phenomenon in patients with obesity [457]. The relatively high mass of the white adipose tissue in these patients causes a lesser sensitivity to insulin, which leads to a reduced storage capacity of new fats in this tissue. As a result, the adipose tissue enhances lipolysis, allowing more triglycerides to be hydrolysed to individual fatty acids, which are then released into the circulation [347,458,459]. Normally, insulin inhibits the lipolysis in the adipocytes of this tissue, but due to the increased mass of the tissue and insulin resistance, this takes place to an insufficient extent [460]. The increase in plasma concentration of the free fatty acids has effects on different tissues. This increase in plasma concentration is accompanied by a decrease in the insulin sensitivity of the muscle and liver. In the liver, insulin normally causes an increase in the gene expression for triglyceride synthesizing enzymes, but simultaneously inhibits the production of very low-density lipoprotein (VLDL) and apolipoprotein B (Apo B), which are involved in the transport of triglycerides, cholesterol and fatty acids into the adipocytes of the adipose tissues [457,461–464]. Moreover, insulin appears to be able to induce the hepatic catabolism of apolipoprotein B [465]. In the situation outlined above it appears that both triglyceride and glucose synthesis and storage as well as the VLDL production are stimulated, causing the release of large amounts of triglyceride-containing VLDL particles by this organ [466]. In addition to triglycerides, the core of the VLDL particles also contain approximately 600 molecules of cholesterol and 1600 molecules of cholesterol esters [467].

Stimulation of the production and secretion of VLDL particles comes at the expense of the hepatic production and secretion of HDL particles, which are involved in reverse cholesterol transport and thus are important for the breakdown and excretion of cholesterol and lipids in the body. Peripheral insulin resistance is therefore mainly characterised by an increased concentration of LDL and a decrease in the HDL concentration in the blood [468–470]. Additionally, the glucose absorption in the muscles is reduced due to the increased resistance to insulin [347,458,471].

Besides the increase in triglyceride synthesis, the production and release of glucose by the liver is also enhanced. In combination with the increased concentration of fatty acids in the blood plasma, this leads to stimulation of the pancreas to release more insulin. However, due to the increasing resistance the glucose and fatty acid levels in the blood remain high, which results in hyperinsulinaemia [472,473]. Research shows that an intravascular injection of insulin has a vasodilatory effect and causes a reduction in blood pressure [474]. This effect disappears later as the protein is capable of stimulating the reabsorption of sodium in the kidney and thereby can increase the osmolarity of the blood. Consequently, the reabsorption of water increases as well, causing the blood volume to increase and the blood pressure to rise again [475]. However, in the case of insulin resistance it appears that the vasodilatory effect is absent, while the stimulatory effect on the sodium reabsorption is maintained [476,477]. In addition, insulin appears to be capable of activating the sympathetic nervous system and causing vasoconstriction [478]. It is suspected that this effect is also preserved in the case of insulin resistance [479]. Altogether, in the case of insulin resistance hyperinsulinaemia may lead to the emergence of hypertension [480–484].

Next to the mechanisms described above, it is found that the adipose tissue of obese patients also exhibits an increased production of pro-inflammatory mediators. Increased plasma concentrations of both interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) could be observed, which contribute to an increased lipolytic activity in the white adipose tissue and further strengthen the insulin resistance of adipose tissue. In addition, increased secretion of these inflammatory mediators into the circulation occurs, causing the insulin resistance of other tissues to increase even further [485–487]. Moreover, the secretion of IL-6 results in an enhanced hepatic glucose production and thus reinforces the hypertensive effects of insulin by indirect stimulation of insulin secretion by the pancreas [488]. The cytokine also stimulates the production and secretion of VLDL, at the expense of HDL production in hepatocytes [489,490]. Another effect exerted by both cytokines is the stimulation of the release of plasminogen activator inhibitor-1 (PAI-1) by the liver and the adipose tissue and the release of fibrinogen by the liver. The release of these substances leads to an increased tendency of blood coagulation in the cardiovascular system and thereby increase the risk of the formation of an embolus [491–493]. Furthermore, obesity seems to lead to a further strengthening of hyperinsulinaemia, increased plasma concentrations of VLDL, glucose and fatty acid and a decrease in HDL concentration [349,489,490,494–498]. Also, the release of pro-inflammatory cytokines by the liver and the adipose tissue leads to an increased inflammatory state of the cardiovascular system, as demonstrated by an observable increase in the plasma concentration of inflammatory marker C-reactive protein [496,499–501].

The hyperglycemic conditions, which occur as a result of insulin resistance and increased hepatic glucose production from fatty acids, prove detrimental to the cells of the endothelium. The damage suffered is the result of an increased production of intracellular free radicals by induction of oxidative stress and modulation of different signal transduction pathways, including signalling cascade of protein kinase C by the increased glucose level in the blood [502–505]. Research involving the administration of vasodilator substances to endothelial tissue revealed that there is a reduced vasodilatory effect under hyperglycemic conditions [506]. In addition, a decreased effect of the endothelium-dependent vasodilation can be observed. This is caused by increased inactivation of nitric oxide by the formation of superoxides as a result of increased oxidative stress [445,507]. While at low concentrations, free radicals can act as signalling molecules in the regulation of cell adaptation and cell growth, high concentrations of these oxidising compounds may lead to damage and apoptosis of cells. In the case of hyperglycaemia the oxidative stress of endothelial and vascular muscle tissue is induced, which increases the risk of suffering damage in these tissues that could lead to the disruption of endothelial functions [445].

Furthermore, the increased oxidative stress caused by protein kinase C activation due to the hyperglycaemic conditions, results in the synthesis of advanced glycation end products (AGE) in the endothelial cells [445]. The AGE molecules are formed by upregulation of the non-enzymatic post-translational modifications of synthesised proteins, making them highly glycosylated [437]. Then, the glycosylated proteins are secreted and can cause damage to the endothelial cells in various ways. First of all, the AGE molecules are capable of stimulating the production of the radicals in the endothelial cells by binding to RAGE (Receptor for Advanced Glycation Endproducts), thereby further increasing the risk of cell damage and apoptosis [508]. Moreover, AGE can interact with different components of the vessel wall, thereby increasing the vascular permeability and expression of adhesion molecules on the endothelium. As a result of the increased endothelial permeability and an increased adhesion of LDL particles and monocytes on the endothelium, an enhanced accumulation of these particles and cells occurs in the subendothelial space underneath the endothelial layer. Normally, VLDL and LDL particles are not harmful to the endothelium, but due to the elevated free radical formation and release of AGE molecules, these particles can be oxidised. Accumulation of these oxidised particles in the subendothelial space can then lead to atherosclerosis. AGE molecules also prove to be able to induce the production of procoagulants by the endothelium, which results in an even stronger coagulation tendency of the blood at this site of the cardiovascular system. In diabetic patients an even stronger increase in the blood sugar level can be observed, resulting in an even stronger enhanced production of AGE molecules and increased oxidation of LDL particles. Due to the increase in influx and uptake from the circulation and oxidation of the LDL particles in the subendothelial space, these patients are at increased risk for the development of atherosclerosis [509–513].

Hyperglycaemia also appears able to stimulate the production of the hormone endothelin-1 in endothelial cells. Release of this hormone by these cells can cause vasoconstriction and induce the proliferation and remodeling of vascular smooth muscle. In addition, the secretion leads to an increased pro-inflammatory cytokine production. The release of these cytokines by the endothelium can contribute to the low-grade inflammation state of the tissue [446].

Also, hyperinsulinaemia appears to be able to contribute to an increase in blood pressure through inhibition of eNOS, which causes inhibition of the production of the vasodilatory nitric oxide. In addition, cultivation studies, in which endothelial cells were exposed to an insulin-rich medium, revealed that insulin is able to stimulate the expression of various adhesion molecules and an enlarged transendothelial migration of neutrophils could be observed. Both of these effects thereby increase the risk for the development of atherosclerosis [514,515].

The development of hypertension can be associated with an increase in angiotensin II formation and activity in the vascular

tissue. Angiotensin II is produced by the endothelial cells and released locally. The hormone has a regulatory effect on a number of functions of the vascular muscle tissue, such as contraction, growth, proliferation and differentiation of these cells [516,517]. An enlarged angiotensin II activity provides an increased expression of the adhesion molecule intercellular adhesion molecule 1 (ICAM-1) and an enhanced release of the monocyte chemotactic protein 1 (MCP-1) by the endothelial cells and can thus promote the influx to and uptake of monocytes into the subendothelial space and thus contribute to development or exacerbation of atherosclerosis [518–520].

In summary it can be stated that the combined and separate influences of the risk factors for the development of cardiovascular diseases lead to disruption of the endothelial functions by increasing the blood pressure, the induction of inflammatory mediators, the stimulation of the blood clotting or induction of the expression of chemotactic substances and adhesion molecules on the endothelium [521–524].

6.3. Atherosclerosis

The previously described metabolic imbalances bring the endothelial tissue in a low-grade inflammatory state, in which various pro-inflammatory cytokines and free radicals are produced by this tissue. The presence of these cytokines causes an increased expression of several adhesion molecules on the endothelial layer, including the lectin-type oxidised LDL receptor 1 (LOX-1). This receptor is responsible for the absorption of LDL particles from the circulation, after which, under the influence of the oxidative stress induced by the metabolic imbalances, oxidises these particles. The oxidised LDL particles (ox-LDL) can then be released into the subendothelial space, but also have several intracellular effects in the endothelium [442,525–527].

Research revealed that ox-LDL can promote the intracellular radical formation and induce the activation of transcription factor NF-κB [528–530]. The transcriptional activity of NF-κB is based on translocation to the nucleus and requires the dissociation of IκB from the heterotrimer. Ox-LDL as well as the pro-inflammatory cytokines stimulate the phosphorylation of IκB, which subsequently causes the heterotrimer to dissociate and the p65/p50 heterodimer to be activated. The free IκB is then degraded in the proteasome. The dimer then moves to the nucleus and affects the gene expression of many proteins that could contribute to the pathological process of atherosclerosis [470]. In this way, ox-LDL stimulates the expression and release of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, and macrophage colony-stimulating factor (M-CSF).

Oxidised LDL itself as well as the pro-inflammatory cytokines and the increase in AGEs then induce the expression of various adhesion molecules, including leukocyte adhesion molecule-1 (LAM-1), intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), as well as P-selectins and E-selectins [525,531–538].

In addition, ox-LDL stimulates the production and release of monocyte chemotactic protein 1 (MCP-1), a chemokine that attracts monocytes, to the bloodstream by the endothelial tissue [539]. Furthermore, ox-LDL enhances the blood coagulation by suppressing the expression and activation of a number of fibrinolytic proteins, thereby increasing the coagulation tendency. This is achieved by inhibiting the expression of thrombomodulin and the activation of the anti-coagulation factor protein C and limiting the release of tissue factor pathway inhibitor and tissue-type plasminogen activator (tPA). The blood coagulation is even further induced by ox-LDL by increasing the production and release of plasminogen activator inhibitor-1 (PAI-1) and tissue factor, a co-activator of blood clotting protein factor VII, in the subendothelial space by the endothelium [540–546].

Both the stimulation of the endothelial production of cytokine TNF-α by the metabolic imbalances and the stimulation by ox-LDL, lead to an induction in the expression of the receptor LOX-1 on the endothelial cells [547–549]. The induction thereby causes an enhanced absorption of LDL particles from the circulation into the subendothelial space, where they are oxidised to ox-LDL due to the oxidative stress of the tissue. Consequently, a feed-forward loop is created, wherein the induction by ox-LDL leads to a stimulation of oxidation and absorption of other LDL particles. This causes accumulation of oxidised LDL in the subendothelial space resulting in an enhanced production of various inflammatory mediators, procoagulent and adhesion molecules, which lead to aggravation of the endothelial dysfunction, stimulation of oxidative stress and the inflammatory state of this tissue [549].

A subsequent significant step in the development of atherosclerosis is the influx of immune cells into the affected endothelial tissue. Due to the inflammatory state, the upregulation of the expression of adhesion molecules on the endothelial tissue and the increased vascular permeability, a large influx of monocytes into the affected tissue can be observed [550–552]. Besides these factors the release of chemokine MCP-1 also promotes the migration of monocytes. The monocytes feature C–C chemokine receptor type 2 receptors, which are able to bind the chemokine and provoke chemotaxis. The monocytes may then adhere to the adhesion molecules on the endothelium, after which extravasation to the subendothelial space takes place [553]. Due to the high concentration of oxidised LDL particles, the monocytes differentiate into macrophages that are able to incorporate the LDL particles and processing these lipoproteins. After processing, the end products are delivered to HDL particles. In this manner, the macrophages constitute a clearance mechanism for the accumulation of triglycerides and cholesterol and try to protect the tissue from the harmful effects of ox-LDL [438].

For the absorption of the oxidized lipoproteins the macrophages express various scavenger receptors, such as CD36 and scavenger receptor type 1 (SR-A1) [554,555]. The affinity of CD36 for ox-LDL is approximately three times higher than for regular LDL [554]. Therefore, CD36 appears to be the primary route of ox-LDL absorption by the macrophages [556]. Absorbed cholesterol esters are hydrolysed in the lysosome to individual cholesterol molecules, that can be incorporated into the cell membrane of the macrophage or converted again to a variety of ester derivatives by acyl-CoA cholesterol acyltransferase-1 (ACAT1) in the endoplasmic reticulum. The reaction products of this enzyme are then, together with triglycerides, phospholipids and other esterified sterols, stored in lipid droplets within the cytoplasm of the macrophage. In this process, a fraction of these lipids is also delivered to HL particles using the ATP-binding cassette transporter-1 (ABCA-1), which is expressed on the cell membrane. Then, HDL-particles filled with lipids are transported to the liver to be degraded. The concentration of cholesterol present in the cell membrane is an indicator for the utilized storage capacity of the macrophage [557,558].

However, in the subendothelial space an imbalance is formed between the absorption of lipids from ox-LDL and the release of lipids to HDL particles. First of all, the metabolic imbalances cause a decreased HDL concentration in the blood plasma, resulting in a reduced secretion capacity of lipids. Secondly, research has revealed that ox-LDL is capable of downregulating ABCA1 by inhibiting the activation of transcription liver X receptor (LXR) in a dose-dependent manner [559]. Consequently, less delivery of the lipids to the HDL particles takes place. Furthermore, ox-LDL stimulates the production and release of the cytokine macrophage colony-stimulating factor (M-CSF), which is able to induce the

expression of SR-A1 on the macrophages, whereby the absorption capacity of the macrophages is enhanced [469]. This shift in the balance between the absorption and secretion of lipids results in the loss of the clearance function of the macrophages. Hence, lipids continue to accumulate in the form of lipid droplets in the cytoplasm of these immune cells. This severe continued accumulation causes the macrophages in the subendothelial space to transform to lipid-rich foam cells and form a plaque inside the vascular wall [438].

In addition to stimulation of the adhesion molecules, receptors and pro-inflammatory mediators, ox-LDL also induces the expression and release of basic fibrolast growth factor (bFGF) and platelet-derived growth factor (PDGF) to the subendothelial space by the endothelium as well as macrophages and smooth muscle of the vascular system. In this way proliferation and differentiation of the smooth muscle cells is stimulated, allowing them to migrate into the subendothelial space [560–562]. Moreover, the activation of this tissue by bFGF and PDGF leads to the production of pro-inflammatory cytokines, procoagulents and metalloproteinases. After migration into subendothelial space, the differentiated smooth muscle cells stabilize the atherosclerotic plaque by forming a fibrous network around the foam cells and ox-LDL particles. In this way, the so-called fibrous cap is assembled [563,564].

The progressive growth of the plaque leads to a reduced dilatory ability of the blood vessel and, together with the simultaneous volume increase of the subendothelial space, leads to narrowing of the blood vessel, which can result in the occurrence of several clinical complications [565,566]. Consequently, ischemia can arise as the resulting obstruction of blood flow leads to shortages of oxygen and nutrients and the accumulation of waste products in the downstream tissues. This complication is called peripheral artery disease and can cause necrosis in these tissues [567].

A second complication develops when the plaque ruptures, causing the accumulated procoagulents and tissue factor to be released into the blood stream and results in the formation of a thrombus. Furthermore, the inhibition of the fibrinolytic systems by dysfunction of the endothelium further enhances this formation. Subsequently, the thrombus can be carried away by the blood flow and cause embolisms and ischemic attacks in remote parts of the body. Most frequently this occurs in the coronary arteries or one of the cerebral vessels. In the first case this leads to the development of a myocardial infarction, while in the second case a transient ischemic attack (TIA) is developed [442–444,568].

6.4. Lipotoxic heart disease

Besides cardiovascular complications that may occur as a result of the disruption of various endothelial functions, disturbance of the lipid metabolism in the heart muscle itself may also lead to complications. Several experiments have found that storage of triglycerides in the myocytes of the heart as a result of high concentration of VLDL particles in the blood, can lead to damage of the myocardium [569]. The heart consumes about ten times its own weight in molecules of ATP per day, and thus has a very high energy requirement [570]. Accordingly, a sufficient amount of energy has to be available in the myocytes at all times in order to comply with changes in the cardiac energy metabolism [571].

This energy is mainly generated through oxidation of fatty acids by the mitochondria of myocytes. When fatty acid oxidation is not sufficient, as can be modulated with the obese Zucker rat, triglycerides accumulate in the myocytes, causing cardiac steatosis, increased retention of fats in this tissue, and can even result in apoptosis [572]. This process is initiated by the infiltration of areas between the myocardial fibres by adipocytes from the epicardium [573]. Then, accumulation of triglycerides in the myocardium occurs, leading to the development of cardiac steatosis. During the development, the storage of triglycerides in lipid droplets can be observed in the cytosol of myocytes. Over time, this phenomenon leads to a reduced contractility of the cardiac muscle [574]. This is though to be caused by the conversion of the triglycerides to ceramide, causing inducable NOS (iNOS) to be activated which results in hypertrophy of the muscle cells [569,575,576]. This process ultimately leads to apoptosis of muscle cells, causing the contractility to be greatly reduced [575,577].

Moreover, PGC-1α appears to affect the mitochondrial metabolism of the heart muscle. Most of the energy is obtained from the oxidation of fatty acids. As described previously, PGC-1α is able to stimulate mitochondrial biogenesis, which could result in an increased mitochondrial capacity enabling the heart to respond to increasing load of the muscle [578,579]. Therefore, stimulation of the PGC-1α activity may be a novel therapeutic opportunity for the treatment of cardiac steatosis and preservation of the heart muscle function. However, *in vivo* studies involving mice revealed that overexpression of PGC-1α in the cardiac muscle tissue leads to an extremely strong mitochondrial proliferation, causing the myofibrils to be moved. These morphological changes of the heart muscle can consequently trigger the development of cardiomyopathy and congestive heart failure [579]. On the other hand, research has demonstrated that inhibition of PGC-1α with histone deacetylase 5 (HDAC5) and cyclin-T1 can cause mitochondrial dysfunction and heart failure, in the case of an increased working load on the heart muscle [580–583].

This effect of PGC-1α was further confirmed in research involving the administration of the beta-adrenergic agonist dobutamine to PGC-1α KO mice hearts. It was found that the ATP synthesis in these mice was not sufficient to meet the increased labour, while wild-type mice were able to comply with the increasing energy demands. This implies that PGC-1α performs an adaptive role for the heart to be able to comply with changes in energy demands during spontaneous labour increase, but is not essential for the basal pump activity [584]. Therefore, PGC-1α contribute to the prevention of heart failure by improving the contractile function when present in physiological concentrations [579,585].

6.5. Preventive effects on heart and blood vessels

Short-chain fatty acids may be able to prevent or counteract a large number of the metabolic disturbances which could lead to loss of endothelial functions and ultimately can result in the development of cardiovascular diseases. From the previously described effects of the fatty acids on the metabolism of lipids and glucose, among others, various preventive mechanisms of action can be derived.

As stated previously, insulin resistance of adipose tissue, which can result of obesity, leads to a reduced fat storage and an increase in the lipolytic activity in this tissue. Due to this increased activity, higher plasma concentrations of free fatty acids arise that reduce the insulin sensitivity in other tissues. In an effort to compensate for the loss of sensitivity to insulin, the pancreas will secrete larger quantities of insulin, which can result in hyperinsulinaemia. Subsequently, hyperinsulinaemia can cause elevation of the blood pressure and thus contributes to the development of cardiovascular complications.

Short-chain fatty acids prove to be able to inhibit the lipolytic activity in the white adipose tissue. To this end, these fatty acids bind to the Ffar2 and -3 receptors present on the adipocytes of this tissue and lead to the activation of intracellular G_i proteins that inhibit adenylyl cyclase. Inhibition of this enzyme leads to subsequent inactivation of the cAMP-dependent PKA and hormone-sensitive lipase (HSL). Consequently, the triglycerides are no

longer hydrolysed to the individual fatty acids. In this way less fatty acids are released into the circulation by white adipose tissue, causing the plasma concentration of the fatty acids to fall [243,316,317,326].

Another mechanism which can contribute to a reduction in the plasma levels of fatty acids is the ability of short-chain fatty acids to induce the fatty acid oxidation in several tissues. Short-chain fatty acids appear to be able to activate 5′-AMP-activated protein kinase (AMPK), which is able to suppress the enzymes involved in the inhibition of the fatty acid oxidation whilst activating the enzymes that induce these oxidations, in both the liver and the skeletal muscles. In addition, AMPK can activate the coactivator PGC-1α which is capable of binding to several transcription factors that are involved in the metabolism of fatty acids. Accordingly, PGC-1α can inhibit the expression of these enzymes at a genetic level, thereby contributing to the stimulation of the fatty acid oxidation.

This way PGC-1α can inhibit or stimulate the expression of these enzymes at the genetic level and thus contribute to a stimulation of fatty acid oxidation. Likewise, the kinase and coactivator can inhibit the hepatic synthesis of fatty acids by inactivation and downregulation of acetyl-CoA carboxylases and fatty acid synthetases [246,252,264].

Additional stimulation of the fatty acid oxidation by AMPK and PGC-1α can occur by promoting the secretion of leptin by the white adipose tissue. It was established that binding of short-chain fatty acids to the Ffar receptors on this tissue caused an increase in the production and secretion of leptin.

Leptin has proved to be able to elevate the AMP:ATP ratio in the liver. As a result, AMPK activation occurs more readily and stimulation of the fatty acid oxidation via this kinase and PGC-1α is further enhanced [266,315,335,586–590]. Moreover, leptin is a potent anorexigenic hormone, therefore restricting the absorption of fatty acids from the ingesta [330–333,591].

Both hypertriglyceridemia and the increased concentration of VLDL particles can provoke the pancreas to release more insulin into the circulation, thereby causing the harmful effects of hyperinsulinaemia on the endothelial tissue. Additionally, the increased release of VLDL particles cause accumulation of triglycerides in the myocytes of the heart as well as steatosis and heart defects [457,461–467].

First of all, these effects are counteracted by the above-described lowering and inhibitive effect of the short-chain fatty acids on the plasma concentration and hepatic synthesis of free fatty acids. Accordingly, fewer amounts of fatty acids are available to be esterified to a glycerol molecule to form triglycerides. Furthermore leptin possesses an inhibitory effect on the hepatic triglyceride synthesis, thereby limiting the synthesis of triglycerides even further [266,334]. It also appears that short-chain fatty acids are able to inhibit the cholesterol synthesising enzymes of the liver via the activation of AMPK, thereby reducing the cholesterol production in this organ [268,430–435]. Besides inhibiting the synthesis of cholesterol, the short-chain fatty acids stimulate the degradation and excretion of cholesterol by the liver. Firstly, this leads to a reduced cholesterol content and production of VLDL particles by the liver [420–422]. Secondly, cholesterol is also capable of inducing the triglyceride synthesis of the liver. Consequently, inhibition of the formation of cholesterol in this organ will also suppress the synthesis of triglycerides [282,436]. An additional effect of the limitation of the LDL and triglyceride synthesis is the secretion of larger amounts of HDL particles by the liver. These particles are involved in the reverse cholesterol transport and thus further contribute to the degradation and excretion of this lipid [420–422].

Hyperglycaemia is the result of an increased hepatic production of glucose from absorbed fatty acids and an increased resistance of the tissues to the actions of insulin, causing the glucose content of the blood to remain high [502–505].

The hyperglycaemic conditions thereby prove detrimental to the endothelium by provoking the development of oxidative stress in these tissues. The formation of superoxides can result in cell damage and hypertension [445,507]. This process also contributes to the development of a low-grade inflammatory state of the endothelium. In this way, hyperglycaemia can contribute to the development of atherosclerosis [446].

As with the reduction of VLDL and triglyceride synthesis, restriction of the plasma concentration of free fatty acids results in a reduced availability of substrates for the production of glucose. Short-chain fatty acids are able to activate AMPK by elevating the AMP:ATP ratio [247,249,252–255]. Next, the activated AMPK can inhibit enzymes that are involved in the gluconeogenesis, including glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) [309–311,346]. Phosphorylation of these enzymes, thereby leads to a reduced production and secretion of the glucose formed by the hepatocytes [309–311]. In addition, short-chain fatty acids are capable of lowering the glucose levels of the blood even more through the activation of the Ffar2 and -3 receptors, which are expressed on the enterocytes of the intestinal tract. Stimulation of these cells via the receptors leads to the secretion of peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) into the portal vein, which is able to reach the systemic circulation after liver passage [358–360,364]. The secretion of PYY leads to improved insulin sensitivity in adipose and muscle tissue. Consequently, more absorption of glucose occurs, thereby decreasing its plasma concentrations [375]. PYY can also suppress appetite by inhibiting NPY neurons in the arcuate nucleus of the hypothalamus and this anorexigenic effect may further limit the intake of glucose [369]. A positive effect on the level of glucose in the blood is achieved by GLP-1 by reducing the production and release of glucagon. This reduction leads to inhibition of the conversion of stored glycogen to glucose, thereby decreasing the plasma concentration of glucose [407,408]. However, GLP-1 could cause adverse effects with respect to endothelial functions as GLP-1 stimulates the pancreas to release more insulin, whilst reducing the secretion of glucagon [398,400,401]. Given that hyperinsulinaemia can exert harmful effects on the endothelium, increased plasma levels of insulin enhance these effects. However, the degree by which GLP-1 stimulates insulin release and the ratio of the increase in insulin release as compared to the amount of release in the case of hyperglycaemia are currently unknown. Hence, it can not be precluded that GLP-1 causes a reduced release of insulin in relation to the amount released when hyperglycaemic conditions exist in the vascular system, by which stimulation of the release of GLP-1 through the enterocytes could still contribute to the reduction of the harmful effects on the endothelium.

Moreover, short-chain fatty acids also appear to exert vasodilatory effects. For example, the intravenous administration of acetate leads to an increase of the cardiac output and an increase in the coronary blood flow. These effects appear to be caused by a direct and an indirect mechanism. First of all, the short-chain fatty acids can activate the Ffar3 receptors present on the vasculature, thereby causing vasodilation [227,592–596]. Secondly, the administration of short-chain fatty acids can stimulate the production of adenosine. The ribonucleoside is a potent vasodilator and is formed by conversion of AMP by the enzyme 5′-nucleotidase [597–601]. The short-chain fatty acids induce the ATP consumption in several tissues, resulting in increases of the intracellular AMP concentrations. The increased availability of substrates for 5′-nucleotidase causes enhanced formation of adenosine and therefore reinforces the vasodilatory effects [599,600]. Due to the reduction of the constriction of blood vessels, the heart is able to pump blood

through the vascular system more easily, hence reducing the risk of heart failure [602]. Moreover, a reduction in blood pressure also results in decreased tendency of blood coagulation, thereby reducing the risk of the occurrence of embolism [603–605]. Furthermore, the lowered blood pressure produces a reduced sensitivity of the endothelium for inflammatory responses and thus lowers the risk for the loss of endothelial functions and the development of atherosclerosis [605–607].

In addition to the vasodilating effects caused by short-chain fatty acids, these fatty acids are also capable of causing vasoconstrictive effects through activation of the Olfr78 receptors, which are present on the vasculature and the renal afferent arteriole [227,229,230]. This effect is achieved by two different mechanisms. First of all, the activation of Olfr78 by acetate or propionate results in the intracellular activation of stimulatory G-proteins. The activation of these proteins antagonises the effects of inhibitory G-proteins and therefore the vasodilatation caused by activation of Ffar3 by short-chain fatty acids. However, it is currently unknown whether both receptors may or may not be co-expressed by one or more subsets of vascular smooth muscle cells. Secondly, activation of the Olfr78 receptors expressed on the renal afferent arteriole induces the release of renin from the juxtaglomerular apparatus, thereby activating the renin-angiotensin-aldosterone system (RAAS). Activation of this system results in an elevation of blood pressure and can therefore counteract the hypotensive effects [230,608,609]. Activation of the Olfr78 receptors in the renal afferent arteriole leads to modulation of the blood pressure within a few hours to days, whereas activation of these receptors on vascular smooth muscle cells can alter the vascular tone in a matter of seconds to minutes [609,610].

However, as described previously, Olfr78 has a lower sensitivity for acetate and propionate than Ffar3. Therefore the compensating effects occur only at higher concentrations of these fatty acids. Several studies report that short-chain fatty acids are found in the circulation in concentrations ranging from 0.1 to 10 mM [611,612]. Because of the lower sensitivity of Olfr78 for acetate and propionate when compared to Ffar3, its compensating effects are only exerted at higher concentrations of these fatty acids. Therefore, the hypertensive effects of Olfr78 feature a buffering character. After all, high concentrations of short-chain fatty acids may result in extreme and potentially dangerous hypotensive responses via stimulation of the Ffar3 receptors. The buffering action that occurs at higher concentrations may therefore counteract excessive drops in blood pressure [608,609].

6.6. Treatment of cardiovascular diseases

Short-chain fatty acids can contribute to inhibition of the development or even to curing of cardiovascular diseases through several mechanisms. In addition to the influence on metabolic processes, the fatty acids also exert an anti-inflammatory on leukocytes and can thereby regulate various immune functions of these cells.

In the case of atherosclerosis, the accumulation of oxidised LDL particles, the low-grade inflammatory status of the endothelium and the release of various pro-inflammatory cytokines and chemokines result in an increased extravasation of monocytes from the circulation to the subendothelium [438,550–552]. At the subendothelium, the monocytes differentiate into macrophages, which can contribute to the uptake, degradation and excretion of the lipids in the ox-LDL particles. To this end, the macrophages express more genes that are involved in the absorption and secretion of lipids. Due to the pro-inflammatory environment the uptake and release of absorbed lipids by macrophages are disturbed, causing the intracellular lipid concentrations to increase and the cells to transform into foam cells [438].

It appears that the altered gene expression of these macrophages can be influenced by various transcription factors, such as the peroxisome proliferator-activated receptors (PPAR) α, -β and -γ. These transcription factors can be activated by activation of PGC-1α [245,246] The strong expression of Ffar2 on the cell membrane of leukocytes enables binding of short-chain fatty acids to the macrophages and facilitates the activation of intracellular AMPK [217,218,224,225]. AMPK then activates PGC-1α, a co-activator for a wide variety of transcription factors, including PPARs [292,438]. Research into the effects of PPARs on inflammatory tissue revealed that the transcription factors were able to suppress the expression of several pro-inflammatory response genes by interfering with pro-inflammatory signalling pathways of activator protein 1 (AP-1) and nuclear factor kappa B (NF-κB) [438]. For example, activation of PPARα leads to inhibition of the induction of adhesion molecules ICAM-1 and VCAM-1 by pro-inflammatory cytokines. This reduces the extravasation of monocytes to affected endothelial tissue, thus reducing accumulation in the subendothelial space [613,614]. Additionally, PPARα activation leads to reduced lipid uptake and storage. The concentration of cholesterol on the plasma membrane is an indicator of the utilized storage capacity of lipids in the macrophage. To this end, cholesterol stored in the intracellular lipid droplets is transferred to the plasma membrane with the aid of, inter alia, Niemann Pick Type C proteins 1 and 2 (NPC1 and -2) [438,615,616]. Activation of PPARα results in stimulation of lysosomal mobilization of cholesterol to the plasma membrane via induction of the expression of NPC1 and NPC2 proteins [617]. The resulting rise in cholesterol levels on the cell membrane of the macrophage causes greater delivery of cholesterol and other lipids to HDL particles. This process is further enhanced through stimulation of the expression of several HDL receptors and the inhibition of ACAT1, which reduces formation and storage of cholesterol derivatives in the macrophages [438].

Apart from the reduced ICAM-1 expression, the activation of transcription factor PPARβ also results in a decreased expression and release of chemokine MCP-1. As a result, a reduced influx and accumulation of monocytes into the subendothelial space could be observed in studies with LDL receptor knockout mice [618].

Research in which monocytes were exposed to PPAR agonists showed that activation of this transcription factor can inhibit both the migration and proliferation of monocytes. PPARγ appears to be able to inhibit the CCR2 expression, thereby suppressing the MCP-1 mediated chemotaxis [619–622]. Moreover, downregulation of the SR-A receptors on macrophages was observed, which resulted into a decreased absorption of lipids [623].

Furthermore, research in which macrophages were incubated with triglyceride-rich lipoproteins has revealed that both PPARα and -γ are able to inhibit the expression of the apoB-48 receptor, thereby reducing the uptake of triglycerides [624]. In addition, both transcription factors induce the β-oxidation of the macrophages through upregulation of CPT-1, which prevents the accumulation of triglycerides, therefore making the macrophages less prone to differentiate into foam cells [438].

Also, both transcription factors were able to inhibit the lateral migration into the subendothelial space as well as the proliferation of smooth muscle tissue. The transcription factors thereby stimulate the expression of cyclin-dependent kinase inhibitor p16, which inhibits the proliferation between the G1 and S-phase of the cell division. In this way, the formation of the fibrous cap is counteracted [625].

Several drugs achieve their effects on macrophages through activation of PPARs. For example, analysis of the CCR2 gene promoter yielded that statins affect the expression of CCR2 on monocytes through the activation of PPARγ. The suppression of the

CCR2 expression by simvastatin appears reversible with the synthetic PPAR inhibitor GW9662 [438,626].

In addition to the anti-inflammatory effects that are achieved through the activation of PGC-1α, both butyrate and propionate feature anti-inflammatory properties [627,628]. For example, after being absorbed, the fatty acids appear capable of suppressing the production of pro-inflammatory cytokines TNF-α, IL-1β and IL-6, whilst stimulating the synthesis and excretion of anti-inflammatory cytokine IL-10 [536,537,629–631]. These short-chain fatty acids also inhibit the expression of VCAM-1 and the chemokine MCP-1, which leads to reduced adhesion and migration of monocytes [470,632].

The anti-inflammatory activity of propionate and butyrate is based on the ability of these agents to inhibit the histone deacetylases (HDAC), causing changes in the expression pattern of various cytokines [633]. Furthermore, the fatty acids interfere with the signal transduction of NF-κB by decreasing the intracellular concentration of p65 as well as by inhibiting the translocation of the p65/p50 heterodimer. Moreover, the short-chain fatty acids stimulate the expression of p50, resulting in an increased formation of p50 homodimers in the cytosol. The formation of the homodimer causes a decreased transcriptional activity of NF-kB, which leads to an even stronger reduction in pro-inflammatory cytokines [634].

The short-chain fatty acids also stimulate the expression of anti-inflammatory cytokine IL-10. Research has demonstrated that release of IL-10 results in a decreased expression of CD36 on the cell membranes of macrophages. Consequently, this lower expression leads to a reduced ox-LDL absorption capacity, thereby counteracting the formation of foam cells [635–637].

Summarizing it can be stated that the short-chain fatty acids inhibit the low-grade inflammatory status of the endothelium by reducing the expression of pro-inflammatory cytokines and adhesion molecules, whilst stimulating the production and excretion of anti-inflammatory cytokine IL-10 [470].

As described earlier in this chapter, PGC-1α performs an adaptive role in the case of changes in energy demands of the heart due to spontaneous labour increase. Induction of PGC-1α could improve the contractile activity of the cardiac muscle cells and can therefore prevent heart failure [584]. However, overexpression of the co-activator leads to cardiomyopathy [585], but possibly a protective effect occurs at physiological concentrations [584]. As short-chain fatty acids can stimulate the activation of PGC-1α via the Ffar3 receptors expressed on the cardiac muscle tissue, the fatty acids might be used to achieve this protective effect of PGC-1α [226]. Especially diabetics could benefit from treatment with short-chain fatty acids, since the PGC-1α expression and mitochondrial capacity are found to be reduced in the heart muscle of these patients [638,639].

7. Discussion

Short-chain fatty acids are formed from nutrients which manage to reach the large intestine by evading the digestive system of the small intestine [3–5]. This distal part of the intestinal tract contains a large and highly diverse population of bacteria, which uses these nutrients for the production of energy needed for growth and preservation of its species. By means of anaerobic respiration and fermentative reactions these substrates are broken down into butyrate, propionate and acetate, in addition to certain gases such as methane, carbon dioxide and hydrogen gas [6,33,34]. The formed products are then released into the lumen of the large intestine and are used by the host for the generation of energy. For example, the short-chain fatty acids are found to contribute significantly to the total amount of formed energy in ruminants [6,9]. However, the contribution of short-chain fatty acids to the total energy requirement of humans is very modest. Nevertheless, the mucosa of the colon appears to predominantly oxidise these fatty acids for the production of energy [36]. Nowadays it is known that about 90% of the secreted short-chain fatty acids is absorbed by the epithelial cells of the intestinal tract [37,38].

Especially the influx of substrates is essential to the growth and preservation of the bacterial population in the colon. Short-chain fatty acids are predominantly formed from a variety non-digestible carbohydrates, such as polysaccharides, oligosaccharides and fructooligosaccharides, but also from proteins, peptides and glycoprotein precursors as well as various endogenous substrates [3,43,640]. The carbohydrate groups that are insensitive to the enzymatic digestion in the small intestine are often called dietary fibres. After reaching the large intestine, the dietary fibres are broken down by a great variety of microbial hydrolases, esterases and lyases to their constituent sugar molecules [49]. The resulting monosaccharides can be classified into two groups, namely, pentoses and hexoses, which consist of five and six carbon atoms respectively. These types of monosaccharides are then converted into phosphoenolpyruvate using different glycolytic pathways [75,76]. The hexoses are degraded by the Embden–Meyerhof pathway, whereas degradation of pentoses occurs via the pentose phosphate pathway [75].

Due to the anaerobic environment of the colon, no oxygen is available to act as an electron acceptor in the reoxidation of NADH to NAD^+ for the formation of ATP. Therefore pyruvate and its derivates are used rather than oxygen. These molecules are partially reduced through various fermentation reactions into short-chain fatty acids, whereafter the end products are released into the lumen of the colon [3,40,75]. Also cross-feeding between bacterial species takes place, wherein one specie supplies fermentable substrates to other bacterial species. In this way, bacterial species that can not breakdown the primary substrates can still sustain in the microbial environment of the large intestine [39,82]. The major end products of the fermentative reactions are acetate, propionate and butyrate. However, other short-chain fatty acids can also be produced, but these agents are often involved in cross-feeding. Therefore, these fatty acids are eventually converted into one of the three major products of the fermentative reactions [73,82].

Numerous factors affect the production pattern of short-chain fatty acids in the colon. First of all, the supply of fermentation substrates delivered to the colon is of importance [11,40]. Moreover, certain types of these substrates are found to result in the formation of a specific end product [44,82]. Secondly, the location of the bacterial species in the colon has an impact on the production pattern. A fierce competition for fermentable substrate exists between the bacterial species. The proximal parts of the intestine contain relatively high concentrations of substrates, therefore allowing more fermentation to occur. Bacteria that are located in the more distal regions of the intestine are less able to form fermentation products due to the lower supply of suitable substrates for these reactions [641].

Another important factor is the microbial composition in the large intestine of the host. This composition shows strong interindividual differences and also changes during the hosts life cycle [40,100,101]. Moreover, it is difficult to identify the full phylogeny of the large intestine using the currently available cultivation techniques as most of the bacterial species are obligate anaerobes that can not survive in an aerobic environment [6]. Nonetheless, it was possible to identify some of the larger bacterial populations and their metabolic properties [6,102].

Research has shown that the apical uptake of short-chain fatty acids *in vivo* occurs with ease and is approximately equal to the absorption of sodium in the colon [110]. Absorption of the fatty acids by the colonocytes from the lumen of the large intestine

presumably occurs via two processes, namely, non-ionic diffusion and transporter-mediated uptake.

The formed short-chain fatty acids feature a carboxylic acid group with a pKa value of about 4.8, whereas in the lumen of the colon an acidic environment exists with a pH value ranging from 5.5 to 6.5. As a result, there is an equilibrium between the protonated, uncharged form and deprotonated, negatively-charged form of the short-chain fatty acids, having between 90 and 99% present in the deprotonated form. For the absorption by non-ionic diffusion it is essential that the fatty acid molecules are present in their uncharged protonated form. To this end, the intestinal epithelium releases protons with the aid of sodium-hydrogen antiporters [38,118–121]. The vast majority of the fatty acid molecules is absorbed by means of trans-membrane proteins, which mediate the transport of negatively charged forms of the fatty acids [121–124].

The first transporter on the apical membrane that can facilitate this absorption is the $SCFA^-/HCO_3^-$ transporter. This transmembrane protein is an antiporter that link the absorption of the negatively charged fatty acid molecules to the release of negatively charged bicarbonate to the lumen of the large intestine, hence facilitating electroneutral transport [127,128]. A second transporter molecule on the apical membrane that contributes to the absorption of the negatively charged fatty acids is the MCT-1 antiporter. This monocarboxylate transporter couples the absorption of the fatty acids to co-transport of protons in the opposite direction, thus facilitating electroneutral transport [135–137,155]. A third transporter on the apical membrane is isoform 1 of the sodium-dependent monocarboxylate transporters (SMCT). The substrate specificity of the transporter is very similar to that of regular monocarboxylate transporters, but it combines the absorption of fatty acids with the co-transport of sodium. The transporter is a symporter that absorbs a fatty acid molecule along with two sodium ions. In contrast to the other apical transporters, this transporter features electrogenic transport instead of electroneutral transport. This absorption activity appears to be driven by an electrochemical sodium gradient across the cell membrane [140,176].

The fraction of the short-chain fatty acids that is not consumed by the colonocytes is able to pass through the basolateral membrane and reach the portal vein [12,22,115,116]. Since the intracellular acidity of the colonocytes is lower than the acidity of the lumen of the large intestine, practically all short-chain fatty acid molecules will be present in the deprotonated, negatively charged form. In this form short-chain fatty acids are highly polar, thus preventing basolateral transport using non-ionic diffusion and making it fully dependent on transporters [110]. The basolateral membrane also features a $SCFA^-/HCO_3^-$ antiporter, which facilitates transport of the fatty acids in the opposite direction. Kinetic analysis of the absorptive capacity of this antiporter revealed differences in the affinity to butyrate relative to the apical transporter, implying that it may concern a different $SCFA^-/HCO_3^-$ antiporter [110,117]. With the aid of immunoblotting studies the presence of MCT-4 and -5 transporters on the basolateral membrane has been demonstrated. MCT-4 transports the fatty acids and hydrogen ions in the opposite direction with respect to the MCT-1 transporter, thus facilitating the release of fatty acids to the portal vein [140,156]. Currently, it is unknown whether MCT-5 is capable of transporting short-chain fatty acids across the membrane. Mapping of the functions of this transporter requires further molecular identification [139,156].

After passage of the basolateral membrane of the colonocytes, the short-chain fatty acids are present in the portal vein which flows into the liver. Prior to reaching the systemic circulation, the fatty acids will have to pass through the liver and escape from its metabolic activities [184–186]. Recently, it was possible to identify two transporters on the membranes of hepatocytes, namely OAT2 and -7, that contribute to the absorption of the short-chain fatty acids.

OAT2 is involved in the uptake of propionate, whereas OAT7 is involved in the absorption of butyrate. Both transporters belong to the sodium-independent, multi-specific organic anion transporters (OAT) [22,187–189]. Other research has revealed that hepatocytes are also capable of absorbing the short-chain fatty acid acetate. However, the applicable mechanism has not been elucidated as of yet [191]. OAT2 is an antiporter that exchanges intracellular dicarboxylates for several extracellular organic anions. The transport process is driven by an inwardly directed dicarboxylate gradient across the sinusoidal membrane [192,193,198,199]. Labelling studies of short-chain fatty acids with mOAT-2 tranfected oocytes have demonstrated that the transporter is only capable of transporting propionate [22].

The OAT7 receptor functions similarly to OAT2, but releases intracellular sulphate conjugates instead of dicarboxylates. For example, in the absorption of butyrate the transporter exchanges sulphate conjugates of the steroid hormone estrone for this fatty acid [187,202,203]. It appears that the liver is capable of converting about 70% of the supplied acetate into other substances. The hepatocytes use this fatty acid not only as an energy source, but also for the formation of acetyl-CoA, long-chain fatty acids and β-hydroxy-butyrate [191,208]. There are records of toxicity due to high blood plasma concentrations of propionate and butyrate. The liver is capable of metabolising large amounts of these fatty acids, which prevents the development of toxic blood levels [211,212]. Assessments of the concentrations of propionate in the portal and hepatic vein indicate that at least 30% of the fatty acid is absorbed by the liver [4,191]. Colonocytes appear to prefer butyrate for the generation of energy. As a result, butyrate is present in low concentrations in the portal vein and the liver appears capable of absorbing and oxidising virtually all butyrate from this vein [242]. Accordingly, only low concentrations of butyrate can be found in the systemic circulation [21,22]. In the hepatocytes, butyrate is subsequently converted to butyryl-CoA and acetyl-CoA [21,22].

Short-chain fatty acids have several regulatory functions on cell metabolism of fatty acids, glucose and cholesterol as well as on immune reactions of immune cells in the microbial environment of the large intestine. These effects are achieved through the binding of the fatty acids to two recently identified GPCRs [28–30]. With the aid of ligand fishing strategy it was demonstrated that GPR41 and -43 could be activated by short-chain carboxylates in a dose-dependent and -specific manner [218,220]. In addition, differences in affinities for acetate, propionate and butyrate appear to exist between the receptors. It was revealed that GPR43 is particularly sensitive to acetate and propionate, whereas GPR41 is predominantly activated by propionate and butyrate [217,218].

After the discovery of these GPCRs, GPR41 and -43 were renamed to free fatty acid receptor 3 (Ffar3) and Ffar2 respectively. The receptors appear intracellularly bound to $G_{i/o}$ proteins, wherein Ffar2 is also coupled to G_q proteins in a similar way [217]. The receptors also feature different distribution patterns among the tissues. It was found that Ffar2 is strongly expressed in various leukocyte populations; in particular monocytes, B-lymphocytes and granulocytes. In addition, a weaker expression of the receptor could be observed in the white and brown adipose tissue, the pancreas, the spleen, the bone marrow and the large intestine [217,218,224,225]. Ffar3 shows a broader expression pattern, featuring the highest mRNA expression of the receptors in the fat tissues. High expressions were also found in the spleen, the pancreas, the lymph nodes and, to lesser extent, on granulocytes and cardiac muscle cells [217,218,225,226].

The fatty acids appear to be able to regulate a large number of immunological functions of leucocytes by using the Ffar2 receptors

expressed on the immune cells. The physiological relevant site for this regulatory effect of the short-chain fatty acids is the highly immunogenic environment of the large intestine. Due to the presence of the enteric microflora, there is a strong need for regulation of the immune cells present in this organ [28–30]. Furthermore, this site features the highest concentrations of short-chain fatty acids, thus enabling very effective regulation of the leukocytes [110,234].

Short-chain fatty acids are capable of inhibiting lipolysis and fatty acid synthesis, whilst stimulating the thermogenesis and oxidation of fatty acids in the body. In this way, the fatty acids can achieve a transition from production of energy-bearing substances to consumption of these substances, causing the plasma concentrations of fatty acids to decrease [243–245]. The short-chain fatty acids affect the fatty acid metabolism by stimulating the activation of AMPK in the liver and muscle tissue. The activation of this kinase leads to an increased expression and activation of transcriptional co-activator PGC-1α, which affects the gene expression of various enzymes involved in the metabolism of fatty acids [246]. In addition, AMPK itself is capable of inhibiting various enzymes that are involved in gluconeogenesis, cholesterol and fatty acid synthesis via direct phosphorylation [252]. AMPK also stimulates, possibly through PGC-1α, the absorption and oxidation of fatty acids in brown adipose tissue, skeletal muscle tissue and liver. Furthermore, short-chain fatty acids can bind to Ffar2 receptors expressed on the white adipose tissue, thereby inhibiting the lipolytic activity and fat storage in this tissue. As a result of these inhibitions, less triglycerides are hydrolysed into free fatty acids, which are then released to the blood. Consequently, the plasma concentrations of the fatty acids are reduced [243,315–317].

Moreover, the activation of AMPK by the short-chain fatty acids leads to a reduction of the glucose content of the blood [244]. Activated AMPK inhibits the hepatic gluconeogenesis via phosphorylation of glucose 6-phosphatase and phosphoenolpyruvate carboxykinase [309–311,345]. Additionally, short-chain fatty acids present in the colon can stimulate the production and release of peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) to the portal vein by binding to the Ffar receptors on enterocytes in the mucosal layer of the intestinal epithelium [357]. The release of PYY results in an improvement in the insulin sensitivity of the liver, skeletal muscle and the brown adipose tissue, which leads to increased glucose absorption by these tissues, therefore reducing the plasma glucose concentrations [34,360,370]. PYY can also regulate appetite and thus limit the intake of glucose-rich food. The release of GLP-1 promotes the production and secretion of insulin, thereby stimulating the absorption of glucose by the three tissues mentioned above even further [403–406].

Several studies indicate that administration of short-chain fatty acids can result in a reduction of the plasma levels of cholesterol [260,282,411–416]. Acetate as well as butyrate and propionate were able to inhibit the synthesis of cholesterol by inhibiting the involved enzymes, namely HMG-CoA reductase and HMG-CoA synthase, via activation of AMPK [424,429]. Furthermore, activated AMPK also reduced the hepatic concentration of HMG-CoA by inhibiting ATP citrate lyase, which leads to a reduced availability of acetyl-CoA for the formation of cholesterol via the mevalonate pathway [282,288]. Another way in which short-chain fatty acids contribute to the lowering of plasma concentrations of cholesterol is through acceleration of the excretion of cholesterol by increasing the production of secretin, causing the hepatocytes to secrete more cholesterol-containing bile [436].

The endothelium of the cardiovascular system appears to play an important role in regulating blood pressure and the free flow of blood through the vasculature. In addition, the endothelium prevents bleeding, can stimulate or inhibit inflammatory reactions and affects nearby vascular muscle tissue in various ways [437]. Complications that can arise in cardiovascular tissues are often due to disturbances in functions of the endothelium. Such disturbances are generally caused by metabolic imbalances and its consequences. For instance, it appears that hyperglycaemia, insulin resistance and dyslipidemia are all able to induce low grade inflammation in tissues and dysfunction of the endothelium [25–27]. These metabolic imbalances can be caused by various disorders, such as obesity, hypertension and type 2 diabetes mellitus, which therefore constitute risk factors for the development of cardiovascular diseases [1,25,438–441].

Endothelial dysfunction by such disorders leads to accumulation of lipids and inflammatory cells in the subendothelial space, which results in a reduction of the inner diameter of the blood vessels. Protraction of this process may result in the development of atherosclerosis; the clogging and hardening of the arteries. This leads to a plaque on the inside of a blood vessel, which hinders the free flow of blood. When the blood vessel is completely obstructed and the downstream tissues are no longer provided with nutrients and oxygen, this will lead in an ischemic attack and may cause necrosis in these tissues [438,442].

In case the atherosclerotic plaque ruptures, large quantities of accumulated procoagulents are released, leading to the activation of the blood coagulation cascade. As a result, a thrombus is formed that can cause blockage of a blood vessel elsewhere in the body. Common sites for the occurrence of an embolism are the coronary arteries of the heart and brain vessels [438,442].

In the case hypertriglyceridemia, accumulation of triglycrides in the cytosol of myocytes of the heart muscle may also occur. This process first leads to hypertrophy and later apoptosis of cardiac muscle cells, which reduces the contractile function of the muscle [443,444,569,575–577].

PGC-1α appears to be expressed in the cardiac myocytes and affects the mitochondrial metabolism of these muscle cells. As the heart muscle mainly oxidises fatty acids for the generation of energy, PGC-1α enables the heart muscle to comply with increased energy demands by stimulating the fatty acid oxidation. Therefore, PGC-1α has an adaptive role in cardiac function and can prevent heart failure due to heavy muscle labour [578,579,584,585]. However, overexpression of PGC-1α in the heart will result in extreme mitochondrial proliferation, causing translocation of the myofibrils which may lead to the development of cardiomyopathy and congestive heart failure [579–583].

Short-chain fatty acids can contribute through various mechanisms to the prevention of the development of cardiovascular diseases and may even be applied in the treatment of these conditions. For instance, the previously described effects of the short-chain fatty acids on the metabolism of lipids and glucose can contribute to the prevention of the development of metabolic imbalances, which are often responsible for the loss of endothelial functions, thus reducing the risk for the development of cardiovascular complications [25–30].

Potential treatments of the cardiovascular diseases with short-chain fatty acids are based on their anti-inflammatory effects on various immune cells. In the case of atherosclerosis, accumulation of LDL particles and macrophages in the subendothelial space occurs. Normally, macrophages are involved in the excretion of LDL particles, but retention of lipids occurs due to a disturbance in the balance between absorption and release of these substances. The increased retention of these substances in the macrophages will lead to differentiation of these immune cells to voluminous foam cells, causing plaque formation and vasoconstriction [438]. Short-chain fatty acids are able to activate AMPK in the macrophages by binding to the Ffar2 receptors present on the membrane of

these cells [217,218,224,225]. The activated AMPK can activate co-activator PGC-1α, which can modify the gene expression of macrophages and thereby restore the imbalance between uptake and release of lipids, thereby counteracting foam cell formation [292,438]. In addition, the activation of PGC-1α reduces the expression of adhesion molecules, therefore limiting the trans-endothelial migration of monocytes from the circulation [613,614].

Moreover, it appears that butyrate and propionate feature anti-inflammatory properties [627,628]. The fatty acids are able to inhibit the production and secretion of pro-inflammatory cytokines TNF-α, IL-1β and IL-6, whilst stimulating the production and release of anti-inflammatory cytokine IL-10 [536,537,629–631]. Modification of the gene expression of these cytokines in the inflammatory cells are caused by the histone deacetylase inhibitory activity of propionate and butyrate, which results in a reduction of the low-grade inflammatory status of the endothelium tissue [633,634].

Altogether, short-chain fatty acids seem to have various beneficial effects on the lipid and glucose metabolism and on the regulation of inflammatory functions of immune cells. Therefore these fatty acids are potentially suitable for the development of new possibilities of intervention in the prevention and treatment of various cardiovascular diseases. However, not all aspects needed for the development of robust therapies have been charted sufficiently as of yet and thus it is remains unclear whether all conditions needed for proper treatment can be met.

For example, there are several publications on the toxic effects of propionate and butyrate, when they are present in high concentrations in the systemic circulation [211,212]. Despite fatty acids being the preferred source for the production of energy by the colonocytes [36], therefore metabolising a large fraction of these substances, and the livers capability to clear virtually all butyrate [37,38], the essential qualitative and quantitative studies on the metabolic capacity and the kinetic order of the metabolism of both the liver and the colonocytes are currently lacking. After all, in the case of stimulation of the formation of butyrate or propionate in the colon, the first-pass effect of these substances is difficult to predict, hence the occurrence of toxic plasma levels can not be ruled out.

Furthermore, it is also unclear whether the plasma levels of short-chain fatty acids can be significantly increased to exert the associated beneficial effects. For instance, in the case of first-order kinetics combined with a high metabolic capacity, an increase in concentration of the fatty acids would result in an enhanced elimination of these substances.

It also appears that overexpression of PGC-1α in the cardiac myocytes and the strong increase in the production and release of GLP-1 by the enterocytes of the large intestine are harmful to the body and can contribute to the development of cardiovascular diseases. It was found that overexpression of PGC-1α resulted in such an extreme increase in mitochondrial biogenesis and capacity that morphological changes of the heart muscle could be observed, which lead to contractile defects and eventually to heart failure [579–585].

GLP-1 secretion from the L cells in the epithelial layer of the colon causes an increase in insulin secretion by the pancreas as to reduce the glucose levels in the blood and thus counteracts endothelial dysfunction due to hypertension [357,398–401].

However, it is currently not known in which ratio the hyperglycaemic conditions and the effects of GLP-1 contribute to the stimulation of the secretion of insulin. Therefore it remains unclear whether the increased release of GLP-1 by short-chain fatty acids has a positive effect on the restoration of endothelial functions or has a detrimental effect by stimulating hyper-insulinaemia, hence contributing to the dysregulation of endothelial functions [398,400,401]. These potentially harmful effects urge for research on the widths of the therapeutic windows of short-chain fatty acids in order to comply with the demands on safety and efficacy of potential treatments.

Apart from oral and intravenous administration of short-chain fatty acids or their salt forms, the use of pre- and probiotics may also be considered for attainment of these beneficial effects. In the case of prebiotics, dietary fibres that selectively promote the growth of a particular short-chain fatty acid-producing bacterial specie are administered in order to increase the production of its fermentation products [642,643]. In the case of probiotics, cultures of micro-organisms are administered, whereafter they can accommodate in the large intestine and produce the favourable metabolic end products [644]. Application of these two methods is impeded at this point due to limitations in the ability to identify the microbial species and their metabolic properties. The composition of the enteric microflora is strongly influenced by a wide variety of influencing factors and can vary from person to person [40,100,101]. The patterns of production of the fermentation products are complex and thereby also subject to interactions between different bacterial species, such as in the case of cross-feeding. As the complete phylogeny, the associated metabolic properties by species and mutual interactions of the species in the colon are not yet mapped, predicting both positive and negative effects is currently not possible [101,102]. Elaborate identification studies of the gut microbiota are essential to assess whether correct application of either pre- or probiotics for the stimulation of the short-chain fatty acid production is feasible.

In summary the following conclusions can be drawn.

1. Cardiovascular disorders are mainly caused by a loss of endothelial functions, or by accumulation of triglycerides in the heart muscle due to imbalances in the metabolism of lipids and glucose [438,442].
2. Short-chain fatty acids are able to restore the imbalances in lipid and glucose metabolism, and thus can make a contribution to the prevention and treatment of cardiovascular diseases [25–30].
3. Butyrate and propionate have the ability to regulate various functions of immune cells and can counteract the low-grade inflammatory status of affected endothelial as to minimise the loss of endothelial functions [217,218,224,225,292,438,613,614].
4. In the case of atherosclerosis, short-chain fatty acids can inhibit the formation of foam cells, the influx of monocytes as well as reduce the production of pro-inflammatory cytokines by the endothelium, and thus contribute to recovery of endothelial dysfunction [536,537,627–631,633,634].
5. For the development of robust therapies with short-chain fatty acids or pre- and probiotics based therapies, in which the production of fatty acids is stimulated, some aspects are still insufficiently mapped, making it unclear whether all preconditions for correct treatment can be met [101,102].

For effective development of interventions for the prevention and treatment of cardiovascular disease based on short-chain fatty acids the following recommendations apply.

1. Research into the metabolic capacity and the kinetic order of the metabolism of short-chain fatty acids in both the liver and the colonocytes.
2. The mapping of the widths of the therapeutic windows of the short-chain fatty acids in order to meet requirements with respect to safety and effectiveness.
3. For the application of pre- and probiotics on based therapies, it is necessary to identify the full phylogeny of the microflora in

the large intestine, the associated metabolic properties of each species and the potential interactions between the species.

Conflict of interest

The authors report no conflicts of interests.

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