REDOX EXPERIMENTAL MEDICINE

REVIEW

High cholesterol diet, oxysterols and their impact on the gut–brain axis

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Abstract

In a Western or Westernized diet, the abundant cholesterol is invariably associated with the presence of biochemically reactive oxysterols, the amount of which mainly depends upon the autoxidation degree of cholesterol itself, during food harvesting, production and storage. Oxysterols, in the average amount and composition detected in a high-cholesterol diet, display remarkable pro-inflammatory and cytotoxic effects on the gut epithelium. Moreover, in a low micromolar range, they may change the physiological level and membrane localization of tight junctions of the intestinal epithelial barrier, which then become leaky and permeable to microbiota. This combination of toxic effects possibly exerted by dietary oxysterols likely contributes to the impairment of the microbiota–gut–brain axis, through both direct and indirect mechanisms hereby reviewed. Importantly, dietary oxysterols are absorbed like cholesterol and circulate in the bloodstream, mainly within LDLs, rendering these micelles more oxidized and dangerous. Last but not the least, dietary oxysterols may deeply interfere with correct gut–brain signalling because of the redox pathways they are hyper-regulating and sustaining.

In conclusion, protective dietary measures should be adopted, including restricted consumption of cholesterol-rich food and reduction of cholesterol autoxidation in food production and storage, for instance by supplementation of food with flavonoids and/or other bioactive substances with strong anti-oxysterol properties.

Introduction

From the unanimous recognition that the connection between the brain and the gastrointestinal tract is bidirectional, a larger and more multidisciplinary investigation has taken place to unravel just how complex and multifactorial are the extraordinarily important crosstalk and mutual influence. The gut–brain axis is not simply based on the vagal nerve connection and on the enteric nervous system but also on a number of signalling pathways triggered and/or regulated by various molecules, including hormones, neurotransmitters, immunological mediators, bioactive molecules and a variety of microbial metabolites (Rhee *et al.* 2009, Mayer *et al.* 2015) (Fig. 1).

Indeed, all communication between the brain and gut occur in the presence of billions of microorganisms normally, but also dynamically, populating the gastrointestinal tract. The gut microbiota certainly play a major and essential role in modulating the gut–brain axis function both in normal and pathological conditions (Lerner *et al.* 2017, Cryan *et al.* 2019, Morais *et al.* 2021). As a straight consequence, any acute or, more frequently, any persistent modification of the gastrointestinal microbiota could considerably impact the gut–brain functional interaction, contributing to impaired mood, memory, learning, general behaviour, response to stressors, the
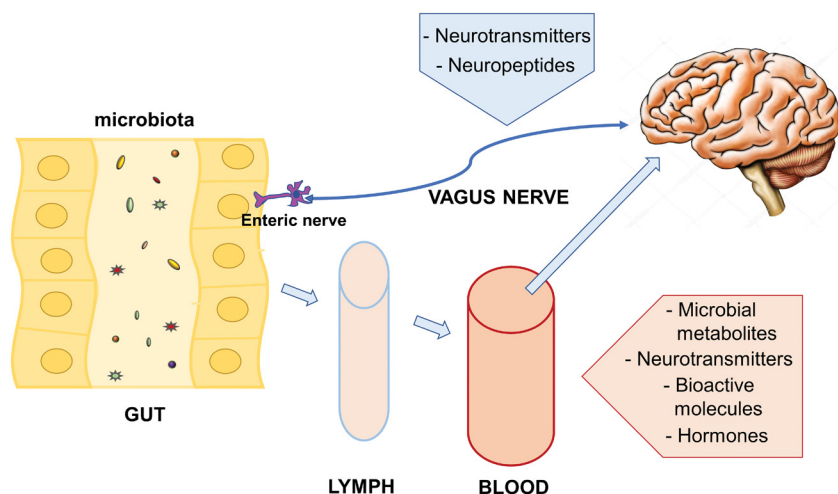
Figure 1
Schematic representation of the gut–brain axis.

worsening of anxious and/or depressive states and, last but not least, to promote neurodegenerative disease processes (Cryan *et al.* 2019, Dicks *et al.* 2021, Morais *et al.* 2021).

Undoubtedly, dietary habit is one of the main factors regulating or dysregulating gut microbiota, preserving or affecting appropriate microbial diversity and composition (David *et al.* 2014, Sonnenburg & Bäckhed 2016) that vary in different periods of life, in particular in early life (Sprockett *et al.* 2018). Among the various types of dietary regimens, the Western pattern diet, rich in cholesterol, saturated fat and n-6 polyunsaturated fatty acids (n-6 PUFAs), is unanimously recognized to be a major trigger and promoter of metabolic syndrome and is considered to favour severe changes in brain structure and function as well (Custers *et al.* 2022).

The deleterious effects of an unbalanced dietary intake of n-6 PUFAs, with their oxidative derivatives, and of saturated fat on the microbiota–gut–brain axis are still being thoroughly investigated (Miao *et al.* 2020, Custers *et al.* 2022), while the likely impact of cholesterol oxidation derivatives on such a functional axis is yet poorly investigated. The quantitatively prevailing and most studied group of cholesterol oxidation products is oxysterols, molecules that differ from cholesterol by having a hydroxyl or carbonyl or epoxy group in the sterol ring or a hydroxyl group in the side chain (Poli *et al.* 2022). Of the wide variety of biochemical and biological properties of oxysterols, we focused here on those that support the possible involvement of this class of compounds in the impairment of the intestinal epithelial barrier and in the consequent impact on the gut–brain axis regulation. Further, the likely but so far scarcely investigated interaction of oxysterols with gut microbiota has also been considered in this review.

Non-enzymatic oxidation of cholesterol present in food

Oxysterols are oxidized products of cholesterol and are generated either enzymatically or non-enzymatically. In the former, the chemical process is mainly rate-limited by the actual amount of the end product that is generated, while in the case of cholesterol autoxidation, because of heat, light exposure, inappropriate storage, freeze-thawing, the process is not regulated and is harder to contain (Poli *et al.* 2022).

The oxysterols of enzymatic origin that are relevant in human pathophysiology are 27-hydroxycholesterol (27OHC), 25-hydroxycholesterol (25OHC), 24-hydroxycholesterol (24OHC) and 7α-hydroxycholesterol (7αOHC), all consistently present in amounts within the nM range in the plasma/serum of healthy individuals (Dzeletovic *et al.* 1995, Pataj *et al.* 2016) and in such a range certainly involved in physiological functions. Common oxysterols of non-enzymatic origin are 7-ketocholesterol (7KC), 7β-hydroxycholesterol (7βOHC), 5,6α-epoxide (α-epoxy), 5,6β-epoxide (β-epoxy) and cholestan-3β,5α,6β-triol (triol), also represented within the nM range in human blood; importantly, 25OHC and 7αOHC are, in part, produced non-enzymatically (Poli *et al.* 2022) (Fig. 2).

The dietary supply of cholesterol and oxysterols already present in foodstuffs and further generated by storage and cooking may become critical for the gut–brain axis, especially in the case of Western or Westernized diet. Of note, cholesterol-containing food ingredients and final products also contain variable amounts of oxysterols of enzymatic origin, but most of the oxysterols introduced by the diet are of the non-enzymatic type, which a physiological role appears difficult to attribute to, even if it
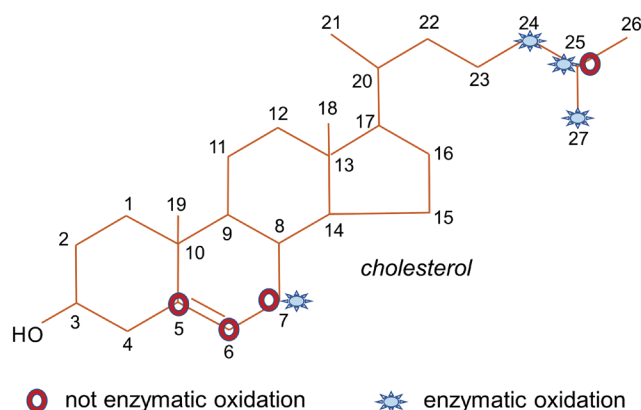
Figure 2
Structure of cholesterol and main sites of oxidation of the molecule.

cannot be excluded *a priori*. One must outline the fact that even the oxysterols of physiological relevance may become harmful when present in excess in a given organ or system. Overall, 7KC and 7βOHC are, by far, the two oxysterols that exert, both *in vitro* and in the experimental animal, the strongest pro-oxidant, pro-inflammatory, pro-apoptotic and pro-autophagic effects (Vejux *et al.* 2020).

Often, the major impact of an excess of non-enzymatic oxysterols occurs in the gut because of a high dietary intake of animal fat that is added to the endogenous cholesterol synthesized mainly in the liver. Consequently, the potential cytotoxicity of a variety of oxysterols vs the epithelial layer of the gastrointestinal tract deserves comprehensive consideration.

Mutual effects between gut microbiota and oxysterols

Microbiota-mediated contribution to the intestinal absorption of cholesterol and oxysterols

Eubiotic gut microbiota remarkably contribute to the metabolism of the cholesterol that reaches the ileum with bile and food (Kriaa *et al.* 2018), by favouring the conversion of part of it into coprostanol, a metabolite rapidly eliminated with faeces. Several bacteria, including *Lactobacilli* and *Bifidobacterium,* are actively operating such conversion (Lye *et al.* 2010*a*). Of the remaining cholesterol in the small intestine, some are likely taken up by the various components of the microbiota (bacteria, archaea, viruses and fungi) for growth requirements (Lye *et al.* 2010*b*); the rest is available for uptake by the intestinal epithelium. This is a complex process, in which the interconnected but inverse activities of the ezetimibe-inhibitable Niemann-Pick C-1 like-1 protein (NPCL1) (pumping in) and ATP-

binding cassette G5 and G8 (ABCG5 and ABCG8) (pumping out), at the enterocyte plasma membrane level, appear to play a major role (Hui & Howles 2005).

The potential contribution of microbiota to the regulation of intestinal absorption of oxysterols of both dietary and endogenous sources has been, so far, poorly investigated. Only very recently, quantification of the effect of a nutraceutical containing *Bifidobacterium longum* on the plasma level of the three main side-chain oxysterols, namely 24OHC, 25OHC and 27OHC, was carried out in a double-blind, randomized, placebo-controlled study. After 12 weeks intervention, no significant difference was observed between placebo and treated group about 24OHC or 25OHC or 27OHC/cholesterol ratio. Only a light, yet statistically significant, reduction (by 10.4%) of 27OHC absolute plasma level was reported (Cicolari *et al.* 2021). Still recently, Lactobacillus was reported to markedly upregulate both the NPCL1 and the ABCG5 membrane transporters at the enterocytic level (Cao *et al.* 2021). Related to this, in a previous study on patients with moderately high LDL cholesterol, 4 weeks' treatment with ezetimibe (a selective inhibitor of the gut transporter NPCL1) led to a marked reduction (by 66%) of the serum level of the highly toxic 7βOHC. On the other hand, no difference was observed in the case of 27OHC and 7αOHC as to the normocholesterolemic controls (Hirayama *et al.* 2013).

Another likely effect of microbiota on oxysterols is the modulation of their amount present within the intestinal lumen prior to any possible uptake by the intestinal mucosa. In fact, intestinal bacteria were demonstrated to upregulate NADPH oxidase 1 (NOX1) and dual oxidase 2, both highly expressed on the brush border of the enterocyte's plasma membrane, in this way increasing the local concentration of oxidant species, in particular, the diffusible oxidant species hydrogen peroxide (Jones & Neish 2017). In this way, further autoxidation of the cholesterol present in the gut lumen may easily occur, thus reasonably increasing the intraluminal amount of oxysterols of non-enzymatic origin.

Oxysterol-mediated inhibition of pathogenic viruses and bacteria

Based on the marked cytotoxic properties displayed by the two oxysterols 7KC and 7βOHC (Vejux *et al.* 2020), some impact by these and related cholesterol oxides on gut microbiota diversity and function should be expected. No relevant studies are available yet, except for one reporting on gut dysbiosis provoked by quite improbable amounts of 27OHC given subcutaneously to C57BL/6J mice (5 mg/kg bwt) daily for 3 weeks (Wang *et al.* 2020).

On the contrary, very lively over the last few years has been the characterization of antimicrobial activity possibly exerted by oxysterols, after the discovery of potent and broad antiviral properties of 25OHC first (Blanc *et al.* 2013, Liu *et al.* 2013), exploited against a variety of pathogenic enveloped viruses, and of 27OHC just afterward, extended also to some pathogenic non-enveloped viruses (Civra *et al.* 2014). Both 25OHC and 27OHC actually target components of the host cells that are essential for viral replication instead of viral structures, by this way strongly reducing the possible onset of drug resistance (Lembo *et al.* 2016). Of particular interest was the *in vitro* demonstration of full inhibition of the intracellular replication of human rotavirus, the most common cause of enteritis in children, as exerted by 27OHC (Civra *et al.* 2014) through the block of viral particles within the late endosomes (Civra *et al.* 2018). Solid proofs of an antiviral effect exerted by oxysterols also *in vivo* are available for 25OHC. In fact, the parenteral administration of this oxysterol to female BALB/c mice or to Rhesus monkeys infected with Zika virus was demonstrated to significantly quench both viremia and disease's severity in both types of animals. Moreover, the 25OHC treatment of pregnant IRC mice infected with Zika virus protected against the virus-associated microcephaly in the fetus (Li *et al.* 2017).

Some antiviral properties, much lower than that of the two side-chain oxysterols, were attributed to other oxysterols, for example, 7KC and 7βOHC, involving gut-unrelated viruses as well (Lembo *et al.* 2016, Ghzaiel *et al.* 2021).

The antimicrobial effects of oxysterols are not limited to viruses. Bactericidal activity of 25OHC was consistently observed by different research groups (for a review see Griffiths & Wang 2021). With regard to two major opportunistic pathogens of the gut, *Listeria monocytogenes* and *Shigella flexneri*, responsible for severe intestinal inflammatory diseases with systemic complications, 25OHC but also 27OHC and 20α-hydroxycholesterol (20αOHC) were demonstrated to markedly inhibit bacterial diffusion from cell to cell, through a rapid remodelling of the cell plasma membrane (PM) cholesterol profile, a condition hampering the PM protrusion process needed for these bacteria to cross the intercellular tight junction apparatus. The three oxysterols were shown able to rapidly mobilize the pool of accessible cholesterol on the cell surface, i.e. not bound to phospholipids, by hyperactivating the acyl-CoA: cholesterol acyl transferase, thus favouring cholesterol esterification and subsequent increased internal localization within the cell (Abrams *et al.* 2020).

Still focusing on the luminal surface of the gastrointestinal tract, it is important to outline that the 25OHC possibly present in the lumen of the gut is essentially of food origin, deriving from the autoxidation of cholesterol-rich foodstuffs. This oxysterol can, in part, be absorbed by enterocytes as for cholesterol (Hui & Howles 2005) and, of interest, may contribute to the impairment of the tight junctions of the intestinal lining as hereafter reported in detail.

Dietary oxysterols and impairment of intestinal barrier integrity

The intestinal mucosa represents the most important barrier able to regulate host response to dietary antigens and maintain appropriate immune tolerance towards commensal bacteria. The gut barrier is not only a mere physical interface separating the inner from the outer environment but also represents a dynamic selective permeable complex system, where different molecules and signalling pathways cooperate in the prevention of harmful luminal antigen penetration and in the regulation of immune and inflammatory responses.

This barrier has a rate-limiting function in paracellular transport and consists of a single layer of intestinal epithelial cells (IECs) firmly anchored to each other by a series of intercellular junctions that constitute the Junctional Complex: transmembrane/cytoplasm Tight Junction (TJ) proteins are in the apical/lateral cell surface, while Adherens Junctions (cadherins and catenin) lay below TJs.

As depicted in Fig. 3, the TJ proteins are (i) junctional adhesion molecules, (ii) tissue-specific occludin and claudins and (iii) TJ plaque proteins like the cytosolic scaffold protein zonula occludens-1 (ZO-1); other small cytosolic and nuclear proteins directly or indirectly interact with TJs. This multi-protein system not only regulates the intestinal cell polarization and the flux of small solutes and bigger molecules from the apical to the basolateral membrane of the cell, but it also modulates intracellular signalling of the immune and inflammatory responses against pathogens.

The association among intestinal barrier impairment, dysbiosis and inappropriate diet composition has raised major interest in several intestinal disorders, including inflammatory bowel disease (IBD). The concept is that 'leaky gut syndrome' and 'dysbiosis' are linked to each other, and that both are involved in gastrointestinal and systemic disorders. Moreover, in industrial-urbanized
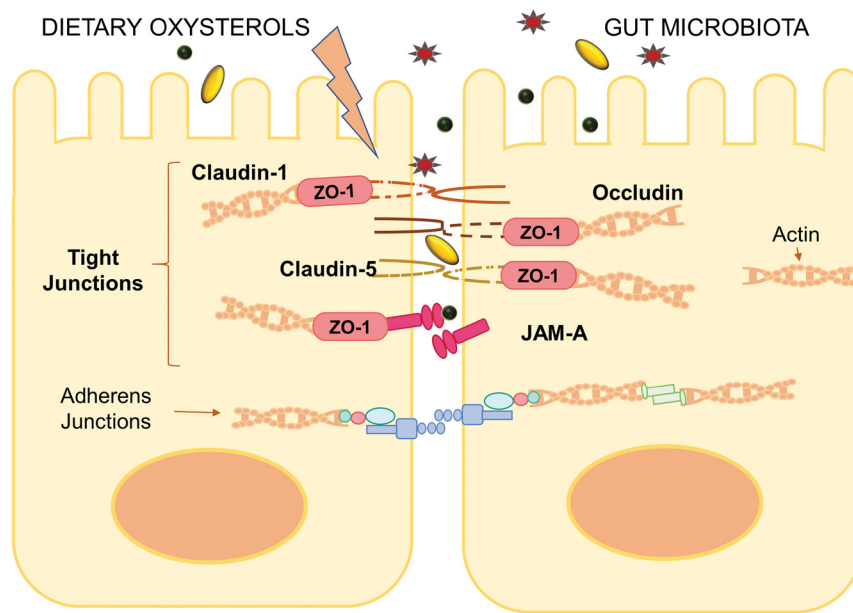
Figure 3
Tight junction impairment by dietary oxysterols and increased permeability of the intestinal lining to microbiota. JAM-A, junction adhesion molecule-A; ZO-1, zonulin-1.

societies, IBD risk appears associated with a Westernized lifestyle, in which the high amount of saturated fat and the paucity of fibres can affect mucosal layer function and intestinal microbe composition with consequent failure of the gut immune response (Statovci *et al.* 2017). Notably, IBD patients often suffer from extra-intestinal manifestations including neuromuscular diseases and psychophysiological vulnerability, likely dependent on a gut–brain axis impairment (Banfi *et al.* 2021).

The interplay of luminal antigens and gut response in triggering inflammatory signals involves pathogen-associated molecular pattern (PAMPs) receptors including the toll-like receptor (TLR) family. The activation of TLR4 and its localization on the enterocyte membrane was proven essential for the gram-negative bacteria-derived lipopolysaccharide (LPS) to selectively increase intestinal TJ permeability both in CaCo-2 monolayer and in the male C57BL/6 mouse (Guo *et al.* 2013). TLRs could be activated not only by microbial ligands but also by many dietary compounds (Yu *et al.* 2010). TLR-recognizing lipids and lipopeptides can also have an affinity for other ligands of lipid origin such as oxysterols. In fact, a mixture of oxysterols representative of a hyper-cholesterol diet, composed of α-epoxy, β-epoxy, 7KC, 7αOHC and 7βOHC, was demonstrated to activate TLR2 and TLR4 in intestinal CaCo-2 cells, by this way inducing the production of inflammatory cytokines such as IL-8, TNF-α and IFN-β (Rossin *et al.* 2019). The same oxysterol mixture was shown to increase the permeability of the intestinal cell monolayer in terms of both alterations of trans-epithelial electrical resistance and TJ protein levels. One of the mechanisms

of action might be associated with TLR induction. The aforementioned dietary oxysterol mixture decreased not only occludin, claudin-1, JAM-A and ZO-1 protein levels in CaCo-2 cells (Deiana *et al.* 2017, Iaia *et al.* 2020), but also strongly impaired occludin, JAM-A and ZO-1 cell localization (Deiana *et al.* 2017).

7KC alone was also described to markedly derange barrier integrity by decreasing trans-epithelial electrical resistance of CaCo-2 monolayer co-cultured on transwell supports with dendritic cells. In this case, a decrease in mRNA expression of ZO-1 but not of occludin was observed (Chalubinski *et al.* 2014). In addition, a similar detrimental effect was found as exerted by 7KC and/or LPS on the endothelial barrier mimicked by human umbilical vein epithelial cells (HUVEC) co-cultured with dendritic cells (Chalubinski *et al.* 2020). Thus, the authors suggested that LPS and 7KC residing in the gut may be translocated systemically and interact by affecting endothelial barrier and immune responses even far from the gut, for example, by favouring atherogenesis. 7KC tripled the permeability of endothelium co-cultured with LPS. It is conceivable that the same could occur as a damage mechanism in neurodegenerative pathologies, considering that an increased tissue concentration of specific oxysterols, including 7KC, 7βOHC and 27OHC (Testa *et al.* 2016), as well as LPS (Zhao *et al.* 2017), was detected post mortem in the brain of Alzheimer's patients.

Particular attention should be paid to the involvement of 25OHC in the modulation of epithelial barrier permeability, despite the contrasting results obtained so far. 25OHC exogenous supplementation ameliorated

the extent of the colonic inflammatory damage and permeability in mice in which colitis was induced by dextran sulfate sodium (Sheng *et al.* 2020). Sheng and colleagues confirmed these results in intestinal CaCo2 and HCT116 cells and suggested a role of cholesterol 25-hydroxylase in preserving ZO-1 and claudin-4 expression and synthesis (Sheng *et al.* 2020). On the other hand, 25OHC and 27OHC were proven to substantially downregulate JAM-A protein levels in other epithelial cell lines, Hela and MA104 (Civra *et al.* 2020), as well as both expression and synthesis of ZO-1 and occludin in HUVEC (Niedzielski *et al.* 2021). While further studies are needed, the role of specific oxysterols in altering the intestinal barrier and their potential involvement in microbial translocation is increasingly evident. Figure 3 illustrates the hypothetic role of oxysterols in altering epithelial barrier permeability, thus allowing bacterial translocation from the gut lumen into subepithelial spaces.

Intestinal absorption and altered hematic profile of oxysterols following a cholesterol-rich diet

Once having highlighted the potential enterotoxicity of oxysterols, especially when accumulated in excessive amount in the intestinal tract, it becomes consequential to linger over the absorption of dietary oxysterols and the possible alteration of their normal hematic concentration. In relation to this important point, just a few studies are available, carried out in experimental models.

Six months of feeding male C57BL/6J mice with a high-fat cholesterol diet (0.15% (w/w) cholesterol as to controls fed with 0.03% (w/w) cholesterol) eventually led to a significant two to three times increase in plasma levels of 7αOHC, 7βOHC, α-epoxy, β-epoxy, 27OHC and ten times increase in 4β-hydroxycholesterol, while 7KC and 25OHC plasma levels, also measured by gas chromatography–mass spectrometry (GC/MS), were apparently unchanged (Wooten *et al.* 2014).

Still in the male C57BL/6J mouse model, a significant increase in 7-hydroxycholestenone and 27OHC plasma levels was detected by HPLC/MS after 4 months of feeding a diet with a cholesterol content ten times higher than that of the standard diet (Guillemot-Legris *et al.* 2016). In the latter study, the plasma concentration of 4β-hydroxycholesterol was consistently found to be reduced as to the lean control along the development of the diet-induced obesity condition, a finding somehow controversial as to that provided by the previous study, quite

different for both experimental protocol and methodology (Wooten *et al.* 2014). Notable was the observation that the amount of 27OHC detectable in the mouse hypothalamus, notwithstanding the high dietary cholesterol loading, was not significantly affected during and at the end of the 4-week feeding treatment (Guillemot-Legris *et al.* 2016).

Conversely, excess cholesterol intake with the diet was reported, in different experimental models, to provoke a significant abnormal accumulation of oxysterols in organs like liver and heart, generating the hypothesis of contributions by cholesterol oxides to the pathogenesis of hepatic and cardiovascular diseases. Indeed, a really marked accumulation of 7αOHC, 7βOHC and 7KC, with a significant increase in 4βOHC, triol and 25OHC as well, but not of 27OHC was detected by GC/MS in the liver of male Wistar rats fed a 1.25% cholesterol-containing diet for six weeks (Serviddio *et al.* 2016). In the heart tissue of male albino rabbits, fed for 2 months a 2% cholesterol-enriched diet, two to three times increase in 7KC, 4βOHC, 25OHC and 27OHC was detected by LC-MS/MS technology (Sozen *et al.* 2018).

Reports on the absorption and tissue localization of oxysterols deriving from foodstuff in humans are few so far.

Six-eight hours after the consumption of a meal rich in oxysterols, including salami and parmesan cheese, a significant increase in 7αOHC, 7βOHC and 7KC plasma levels was detectable in five male volunteers, with a prevalence of 7βOHC as to the other oxysterols in chylomicrons (Linseisen & Wolfram 1998). A differential efficiency rate in the intestinal absorption of dietary oxysterols appeared evident, as previously reported in detail in a rat model (Osada *et al.* 1994).

An indicative, yet small, kinetics analysis was performed in six human volunteers fed a standard meal supplemented with 400 mg of α-epoxy. The serum content of this tracing oxysterol was then monitored at 2, 4, 8, and 10 h and in three individuals also after 24, 48, and 72 h. The α-epoxy content in chylomicrons and residual chylomicrons peaked at 2–4 h after the meal, while the sterol's content in LDL and HDL showed a peak at 8 h but still present in the circulation within those micelles well after 72 h. Of note, the measurable amount of α-epoxy in VLDL was consistently very small (Staprans *et al.* 2003). Such a study certainly provides further proof, this time in humans, that oxysterol circulation in the blood stream occurs together with cholesterol.

A clear and comprehensive review analysis of intestinal absorption and hematic circulation of dietary oxysterols in the different types of lipoprotein micelles was recently provided by Zmysłowski and Szterk (2017),

confirming that cholesterol oxides are for a brief period mainly concentrated in chylomicron remnants, then for several days in LDL and HDL. A careful insight in the likely contribution of oxidized LDL in the early pathogenesis of atherosclerosis was also provided (Zmysłowski & Szterk 2017). In Fig. 4, actual knowledge about absorption and circulation of dietary oxysterols is schematically depicted.

Dietary oxysterols and gut–brain cell signalling

As far as the impact of dietary oxysterols on the gut–brain axis is concerned, a key point is to elucidate how they could convey perturbing signals, mainly of the pro-inflammatory type, across the blood–brain barrier (BBB). At least some members of the oxysterol family can directly cross the BBB. This is true for 27OHC, of which a low but consistent flux from the circulation into the brain was, for the first time, proven by a careful study on healthy male human volunteers (Heverin et al. 2005). In the same report, the transfer of extracerebral 27OHC into the brain was confirmed in a rat model, in which, however, the entity of the flux through the BBB was proven to be significantly lower than in humans, likely because the blood content of this oxysterol is much lower in murine models (Heverin et al. 2005). In humans, the slow but net flux of 27OHC towards the brain occurs along a concentration gradient mainly determined by the physiologically valid cerebral metabolism of this oxysterol (Björkhem et al. 2019).

At least in people with a Western or Westernized diet, one should expect an increased concentration gradient through the BBB for oxysterols other than 27OHC, especially for 7βOHC and 7KC, quantitatively more highly represented in those types of diet.

In addition, oxysterols reaching potentially toxic concentrations in the blood could be able to affect the BBB endothelial barrier as they do in the case of the intestinal epithelial layer. Compounds like 7KC appear to contribute to the endoplasmic reticulum stress induced by oxidized

LDL in the endothelial lining of arteries, by contributing to the early pathogenic phases of atherosclerosis (Luchetti et al. 2017). A similar effect may be exerted at the BBB level by 7KC and other oxysterols stemming from autoxidation of dietary cholesterol, but this is yet to be demonstrated. Very recently, 7KC was reported to induce marked cytotoxic effects in vitro on human CMEC/D3 brain endothelial cells including the impairment of tight junction localization and the induction of several pro-inflammatory cytokines; however, the oxysterol concentration shown to be effective was actually quite high (Koh et al. 2021). Further investigations are thus needed to validate the hypothesis of a possible derangement of the BBB by oxysterols as depicted in Fig. 5.

On the contrary, dietary oxysterols may undoubtedly trigger even more remarkable gut–brain signalling through a net upregulation of enterocyte expression and synthesis of pro-inflammatory cytokines, molecules indeed recognized as able to directly cross the BBB (Banks 2001, Cryan et al. 2019). The challenge of a differentiated human intestinal CaCo-2 cell line with a representative mixture of oxysterols that are present in processed and/or stored cholesterol-rich foodstuff (30 µM in total) led to both strong stimulation of NOX1 and a derangement of the mitochondrial membrane potential, with a consequent net increase in intracellular ROS production (Biasi et al. 2009).

An identical oxysterol mixture was then demonstrated to markedly upregulate the expression and synthesis of the pro-inflammatory cytokine interleukin (IL)-8 (Mascia et al. 2010). Of note, among the different components of the mixture, 7βOHC was by far the most effective, being also able to exert a significant increase in IL-8 synthesis when added alone to the cell incubation medium at 4.4 µM final concentration. The same authors then showed the oxysterol mixture as able to upregulate in differentiated CaCo-2 cells the expression of other inflammatory cytokines, like monocyte chemotactic protein-1, IL-6 and IL-23 (Mascia et al. 2010).

Some pro-inflammatory cytokines, including IL-6, were demonstrated to pass through the BBB by means of active transporters in an amount apparently sufficient
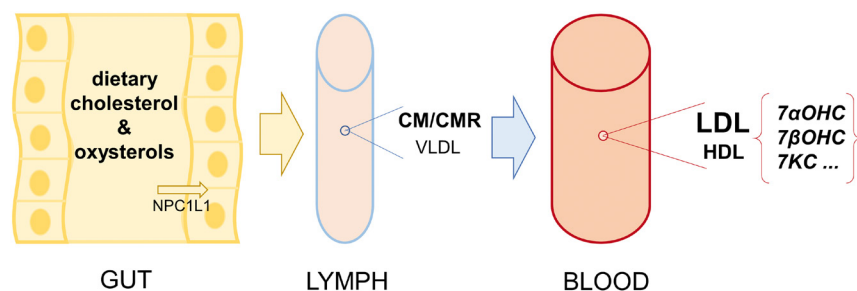
Figure 4
Gut absorption and blood circulation of dietary cholesterol and oxysterols. CM, chylomicron; CMR, chylomicron remnant; NPC1L1, Niemann-Pick C1-Like1 transporter; VLDL, very low-density lipoprotein; 7αOHC, 7α-hydroxycholesterol; 7βOHC, 7β-hydroxycholesterol; 7KC, 7-ketocholesterol.
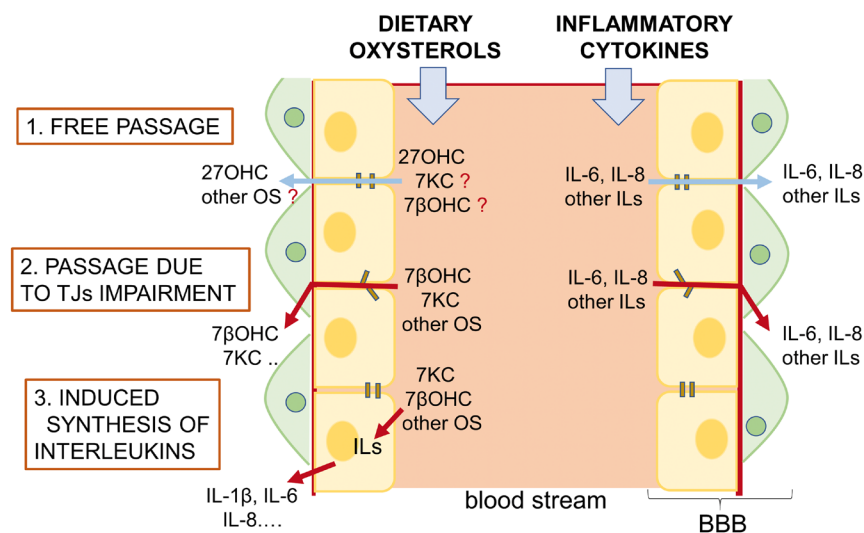
Figure 5
Fluxes of dietary oxysterols and gut-derived inflammatory cytokines through the blood–brain barrier. BBB, blood–brain barrier; ILs, interleukins; IL-6, interleukin 6; IL-8, interleukin 8; IL-1β, interleukin 1β; OS, oxysterols; TJ, tight junctions; 7KC, 7-ketocholesterol; 7βOHC, 7β-hydroxycholesterol; 27OHC, 27-hydroxycholesterol .

to impair brain functions (Banks 2001), for instance, to contribute to the pathogenesis of anorexia (Banks 2001) and of various depressive disorders (Ting *et al.* 2020). These latter conditions are often associated with impaired gut permeability and dysbiosis (Morais *et al.* 2021).

Finally, at the level of BBB, various vascular stimuli may induce endothelial cells to synthesize pro-inflammatory cytokines and release these molecules on the cerebral side (Banks 2006). Even if not yet directly demonstrated, this event appears likely to occur also under the stimulus of excess hematic levels of oxysterols largely deriving from the diet (Fig. 5).

Conclusions and perspectives

In a dietary regimen rich in cholesterol, there is no doubt that a significant amount of potentially toxic oxysterols is consistently present. Their actual concentration mainly depends on the degree of cholesterol autoxidation occurring during production, storage and cooking of foods of animal origin, and, even if a threshold for their toxicity has not been established yet, the experimental evidence achieved so far clearly and unanimously indicates that an oxysterol mixture in the low micromolar range is most likely harmful.

Oxysterols have been demonstrated as able to affect, at the intestinal level, the correct amount and localization of various protein components of the tight junction complex, with consequent increased permeability of the epithelial barrier to the microbiota and to opportunistic pathogens in particular. Together with the microbiota, and maybe interacting with it, oxysterols might thus induce a persistent inflammatory state in the intestinal mucosa that in turn would impair the function of the gut–brain axis. Indeed, the combination of pro-inflammatory substances stemming in excess from an inflamed gut and pro-inflammatory oxysterols, which are easily absorbable with cholesterol and circulating with it in the blood stream, represents a molecular weapon at the systemic level. The brain is included in this risk of damage, because of the ability of defined interleukins and oxysterols to cross the BBB.

Besides a reasonable restriction of cholesterol-rich food consumption, another promising protective measure against the possible impairment of the microbiota–gut–brain axis by dietary oxysterols should be the reduction of cholesterol autoxidation in food production and storage by different means, including a suitable addition of flavonoids and/or even methylxanthines. Actually, the broad pro-inflammatory effect exerted on differentiated CaCo-2 intestinal cells, challenged with the dietary representative oxysterol mixture mentioned above, was strongly or even fully prevented by cell pre-incubation with epigallocatechin-3-gallate (Mascia *et al.* 2010), epicatechin (Deiana *et al.* 2017), extra virgin olive oil phenolic extract (Serra *et al.* 2018), theobromine (Iaia *et al.* 2020) and cocoa bean shell, a mixture of flavonoids and methylxanthines (Rossin *et al.* 2021). Indeed, the presence of a suited amount of these redox active nutraceuticals in the diet appears recommendable, particularly in clinical conditions of actually diagnosed leaky gut.

not involved in the review or editorial process for this paper, on which they are listed as authors.

Author contribution statement
G P and F B conceived and wrote the paper; N I carried out the search and critical analysis of the literature and supervised the technological aspects; V L provided significant contribution to the preparation of text and figures of the revised manuscript; G P designed the illustrations.

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