Chapter

Beneficial Effects of Proanthocyanidins on Intestinal Permeability and Its Relationship with Inflammation

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Abstract

The intestinal barrier is constantly exposed to potentially harmful environmental factors including food components and bacterial endotoxins. When the intestinal barrier function and immune homeostasis are compromised, inflammatory conditions may be developed and impact overall health. Evidence from experimental animal and cell-culture studies suggests that exposure of intestinal mucosa to proanthocyanidin-rich plant products may contribute to maintain the barrier function and to ameliorate the inflammation present in prevalent pathologies such as diet-induced obesity and inflammatory bowel disease. In this review, we aim to update the current knowledge on the bioactivity of PACs in experimental models of altered intestinal permeability and in humans, emphasizing the beneficial effects of grape-seed proanthocyanidin extracts in intestinal health and giving insights into the subjacent biochemical and molecular mechanism.

Keywords: gut, permeability, inflammation, metabolic endotoxemia, obesity, IBD, flavonoid, flavan-3-ol, condensed tannin, procyanidin

1. Introduction

The primary function of the intestinal tract is to digest food components and absorb nutrients and water from the lumen to the systemic circulation. The intestine is also a physical barrier that is in contact with the environment. As a result, the intestinal epithelium is constantly exposed to potentially pathogenic microorganisms, toxins, and harmful components of the diet. When there are disturbances in the barrier function and mucosal immune homeostasis, the influx of intestine luminal content triggers barrier dysfunction and an exaggerated mucosal immune response [1]. Ultimately, chronic exposition to these detrimental environmental stimuli may lead to the development of local and systemic inflammatory conditions [2, 3] that contribute to barrier dysfunction.

Natural products have been recognized as a source of therapeutic agents for many years [4]. Some plant-derived phenolic compounds show promising

anti-inflammatory effects and have been associated with the prevention of certain chronic diseases [5]. Proanthocyanidins (PACs), also known as condensed tannins, are oligo- and polymeric end products of the flavonoid biosynthesis pathway in plants [6]. There has been extensive laboratory research into the effects of both pure PAC molecules and PAC-rich extracts on overall health. These phytochemicals show a wide range of physiological activities [7], including anti-inflammatory and barrier-protective effects in the intestine [8–10], which may be interesting in the context of diet-induced obesity and inflammatory bowel disease (IBD).

We have reported previously that grape-seed PACs and other flavonoids have beneficial effects on inflammation [11–13] and protect the intestine against alterations associated with diet-induced obesity in rats [8, 9, 14, 15]. In addition, research conducted during the last decade with cell culture and animal models has made significant progress in determining the underlying mechanism of the health-promoting properties of PACs in the gastrointestinal tract and peripheral tissues.

2. Altered networks in intestinal dysfunction: barrier integrity and inflammatory response

The intestinal epithelium is a single cell-layer responsible for separating underlying mucosal tissues from the environment and is the largest exposed surface area in the body [16]. As there is a prolific commensal microbial community in the intestinal lumen (intestinal microbiota), epithelial integrity plays a pivotal role in maintaining overall health [16, 17]. The intestinal epithelium is integrated by several cell types with specialized functions. The enterocytes are responsible for the absorptive function and constitute the most abundant epithelial cell lineage. The goblet cells are implicated in the synthesis of secretory mucin glycoproteins that form the mucus layer [18]. Other cellular types integrating the epithelium, microfold (M) [19], Paneth and enteroendocrine cells are specialized in antigen sampling and presentation to dendritic cells, synthesis of antimicrobial peptides, and secretion of hormones, respectively.

The first strategy the host tissue has to maintain its homeostatic relationship with the intestinal microbiota is to minimize the physical interaction with microorganisms, thus limiting microbial translocation and physiological inflammation [20, 21]. The thick mucus layer secreted by goblet cells represents a primary defense line against environmental insults [18]. In addition, the enterocytes are joined together forming an intricately and well-regulated barrier sustained by intercellular junctions linked to the cell cytoskeleton, such as tight junctions (TJs), desmosomes, and adherent junctions. TJs partially seal the paracellular space and prevent passive transport of large molecules, including microbial components and other potentially harmful agents [1, 22].

The paracellular and transcellular pathways are the two major pathways mediating transmembrane transfer of intestinal bacterial substances. Both mechanisms may be involved in intestinal mucosal barrier damage and bacterial translocation. The paracellular pathway is integrated by tight junctions (TJs), consisting of zonulin/zonula occludens (ZO)-1, occludin, claudins, junction adhesion molecules (JAMs), and actin-myosin cytoskeletal proteins. Previous studies have shown that inflammatory cytokines and bacterial antigens can affect the expression level and assembly of these elements, thereby exerting an influence on TJ functions [23]. Immune cells, including neutrophils, dendritic cells, and monocytes, have also been directly implicated in inducing disturbances in TJ barrier function. It has been postulated that pro-inflammatory cytokine-induced opening of the intestinal TJ barrier is an important mechanism contributing to the TJ barrier defects present in

various inflammatory conditions of the gut [24]. Previous studies [25–28] have shown that myosin light chain kinase (MLCK) plays a central role in the regulation of intestinal TJ permeability. The activation of MLCK catalyzes the phosphorylation of myosin light chain (MLC), inducing contraction of the peri-junctional actin-myosin filaments and the opening of the TJ barrier. In contrast, inhibition of MLCK activation prevents this effect [27]. It has been suggested that the cytokine-mediated barrier dysfunction could be mediated by an increase in Nuclear Factor (NF)-kB, which, in turn, activates MLCK gene and protein expression [29] (**Figure 1**).

Once intestinal bacteria and endotoxins enter the portal vein and/or lymphatic system, they can reach other tissues and organs, leading to a cascade response modulated by inflammatory mediators. This situation can induce a systemic inflammatory response, which further damages the function of the intestinal barrier [30]. The endotoxin-signaling pathway includes the binding of LPS to LPS-binding protein (LBP) and its subsequent transfer to the CD14 receptor. LBP-bound LPS initiates inflammation via TLRs associated with membrane-anchored CD14 [31]. TLRs are a family of pattern-recognition receptors that play a key role in the innate immune system. Among all, the TLR4 is expressed at high levels in the intestinal tract, and given that LPS is its specific ligand, TLR4 could be considered the first barrier for recognition of bacterial presence in the gastrointestinal tract. NF-kB is the final effector transcription factor of the TLR4 signaling pathway. It promotes the development of many intestinal diseases and also plays a pivotal role in the translation and transcription of inflammatory mediators [30].

In mammals, the NF-kB family comprises five proteins, including p65 (RelA), RelB, c-Rel, p105/p50 (NF-kB1), and p100/p52 (NF-kB2), which associate with each other to form transcriptionally distinct homo- and heterodimeric complexes; the p65:p50 heterodimer is the most abundant and the most relevant for inflammation [32]. In resting cells, the p65:p50 NF-kB heterodimer is sequestered in the cytoplasm by binding to its inhibitory protein, IkappaB (IkB). In response to an inflammatory stimulus, such as LPS, the classical NF-kB activation pathway leads to the activation of the IkB kinase (IkkB), a member of the IKK complex, triggering IkB-a phosphorylation (pIkB-a). Then, pIkB-a is recognized by the ubiquitin ligase machinery, resulting in its polyubiquitination and subsequent proteasomal

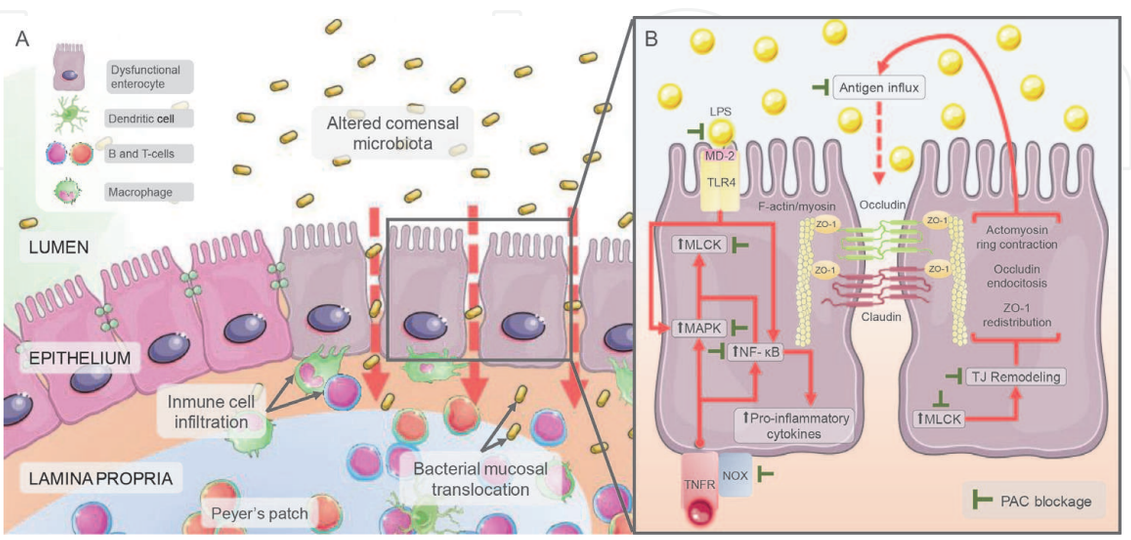


Figure 1.
Protective properties of PACs in the intestinal barrier function. (A) Chronic exposition to detrimental environmental stimuli may lead to dysbiosis, breakdown of the intestinal barrier, influx of bacterial endotoxins and mucosal inflammation. (B) PACs ameliorate loss of barrier function blocking the activation of MLCK mediated by NF-κB and MAPK signaling. See text for details.

degradation. After pIkB-a degradation, the p65:p50 heterodimers are able to translocate to the nucleus, where they bind to the kB motif found in the promoter or enhancer regions of numerous pro-inflammatory genes to induce their expression [33].

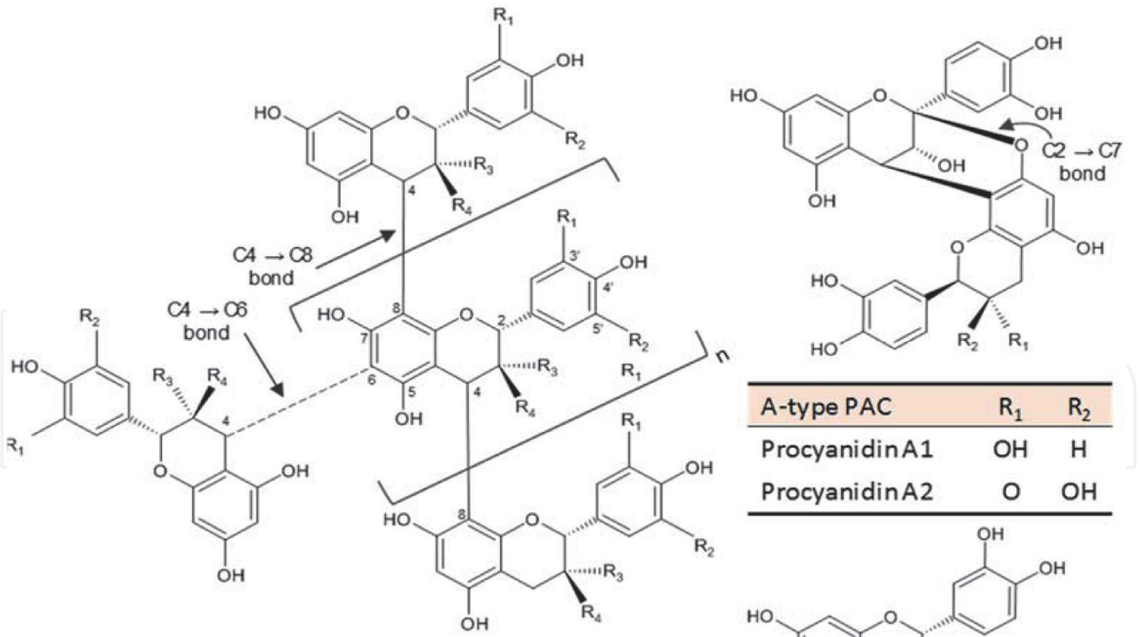
NF-kB target genes include cytokines (e.g., tumor necrosis factor (TNF)-α and interleukins), adhesion molecules, acute phase proteins, and inducible enzymes (inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2)), among others [11]. All of these genes contain verified NF-kB binding sites in their sequences, providing strong experimental evidence for their direct control by NF-kB [34]. Among all of these genes, the expression of iNOS and COX2 has been widely studied in relation to intestinal inflammation. In this regard, sustained high nitric oxide (NO) production by iNOS plays a role in the pathology of chronic inflammatory bowel disease [35, 36]. During the last decade, it has become increasingly clear that NO overproduction by iNOS is deleterious to intestinal function [37], thus contributing significantly to gastrointestinal immunopathology. Cyclooxygenases are enzymes that are responsible for the metabolism of arachidonic acid, converting it into prostaglandins. These products influence a wide variety of biological processes, ranging from homeostasis to inflammation [38]. There are two cyclooxygenase isoforms: the constitutive COX1 isoform and the inducible COX2 isoform [38, 39]. As a result of COX2 induction, prostaglandin E2 levels increase at the site of inflammation and can also be detected systemically.

Multiple environmental factors have been identified as potential triggers of intestinal inflammatory conditions, including Western dietary habits [40]. It has been described that saturated fats play a direct role in inflammatory signaling. Saturated fatty acids (SFA) such as lauric (C12:0) and palmitic (C16:0) directly induce NF-κB activation, acting as non-microbial TLR2 and TLR4 agonists in macrophages [41]. Data suggest that activation of TLRs by SFA is mediated by TLR dimerization and recruitment into lipid rafts [42]. We have reported mild intestinal inflammation and increased permeability in rats feeding on a cafeteria diet consisting of high-saturated fat/high-refined sugar food products [43]. This enhanced permeability has been shown to favor bacterial LPS and other potentially pro-inflammatory molecules entering the systemic circulation, which is known as metabolic endotoxemia [15].

Taken together, these data suggest that HF diet-induced changes in the intestinal microbiota could be responsible for metabolic endotoxemia and for the onset of the corresponding diseases. The causative link between changes in intestinal bacteria populations, endotoxemia, and metabolic disease needs further assessment [44], but the mechanisms likely include altered epithelial permeability, translocation of bacterial products, and upregulation of pro-inflammatory cytokines and hormones produced by gut endocrine cells, mechanisms which might be modulated by PACs.

3. PACs: chemical structure, occurrence, and intake

PACs consist of flavan-3-ol subunits with a degree of polymerization (DP) equal to or greater than 2, mainly linked by (4 → 8) or (4 → 6) carbon-carbon bonds (B-type PACs) [45]. In some botanical sources an additional (2 → 7) ether-linkage also occurs (A-type PACs) [46] (**Figure 2**). Depending on the type of monomers, PACs can be classified into procyanidins, prodelphinidins, and propelargonidins. The most abundant group, procyanidins, consists exclusively of (+)- catechin and (−)- epicatechin monomers [47]. Prodelphinidins and propelargonidins are composed of (−)- gallocatechin/(−)- epigallocatechin and (+)- afzelechin/(−)- epiafzelechin monomers, respectively [45], and have a more limited distribution



Flavan-3-ol monomer	R_1	R_2	R_3	R_4
(+)-Catechin	H	OH	H	OH
(−)-Epicatechin	H	OH	OH	H
(+)-Gallocatechin	OH	OH	H	OH
(−)-Epigallocatechin	OH	OH	OH	H
(+)-Afzelechin	H	H	H	OH
(−)-Epiafzelechin	H	H	OH	H

A-type PAC	R_1	R_2
Procyanidin A1	OH	H
Procyanidin A2	O	OH

B-type PAC	R_1	R_2
Procyanidin B1	H	OH
Procyanidin B2	OH	H

Figure 2.
Chemical structures of PACs. Flavan-3-ol monomers differ based on the hydroxylation pattern and their cis- or trans-configuration. Dimers A1/A2 and B1/B2 are shown as example of A- and B-type PACs, respectively.

Dietary assessment studies have shown that PACs, especially procyanidins are among the most abundant polyphenols in the human diet [6], as they are present in a variety of botanical sources and plant food products such as tea, fruits, nuts, cacao products, legumes, and cereal grains [1, 2]. However, PAC intake varies widely between geographical regions and cultures and is greatly dependent on eating habits, lifestyle behaviors, and socioeconomic status [48]. The daily PAC (dimers to polymers) intakes in adult populations from Korea, the U.S., Mexico, and EU were estimated as 71 [49], 73 [48], 103 [50], and 123–180 mg [51, 52], respectively, but intakes up to 230 mg d^{-1} have been reported in some regions of Spain and Norway [53].

4. The fate of PACs after ingestion

Flavan-3-ols are remarkably stable during gastric transit in humans [54]. Monomers such as (+)- catechin and (−)- epicatechin are readily absorbed in the upper sections of the small intestine [55, 56], recognized as xenobiotics and then subjected to an extensive phase II metabolism that generates glucuronidated, sulfated, and methylated conjugates [57]. Flavan-3-ol monomers and their conjugated metabolites reach peak plasma concentration 1–4 h after flavan-3-ol-rich food consumption [58–60]. Studies conducted in cultivated epithelial monolayers [61–63], rats [64, 65], and humans [60, 66] indicate that PAC absorption is conversely more

limited and is highly dependent on DP, and that the permeation of larger oligomers (DP > 5) and polymers is negligible. No PAC transporter has been identified in the enterocyte membrane in the small intestine. Thus, dimers to tetramers are passively transported across the intestinal epithelium essentially by paracellular diffusion. Although transcellular passive diffusion is not likely to occur due to the hydrophilic nature of PACs conferred by the multiple hydroxyl groups, uptake might be possible by endocytic mechanisms [62].

In humans, a study assessed the contribution of the ingested cocoa flavan-3-ols and procyanidins to the systemic pool, and found that the plasma (−)- epicatechin came from the orally administered cocoa (−)- epicatechin and not from their oligomers or polymers [67]. This is in agreement with the evidence obtained with rats that suggests that PACs from different sources do not depolymerize to monomers after ingestion [68, 69]. Stalmach et al. [56] conducted a study with ileostomized patients who were administered green tea, and found 70% of the ingested flavan-3-ol in the ileal fluid after 24 h. Altogether, these findings suggest that substantial amounts of ingested flavan-3-ol monomers and PACs remain unabsorbed in the small intestine and reach the colon. There, they are efficiently transformed by the colonic microbiota into low molecular weight phenolic compounds that can be absorbed by colonocytes [57].

In vitro fermentation of purified procyanidin dimers with human fecal microbiota has shown to produce mainly 2-(3′,4′-dihydroxyphenyl) acetic acid and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone [70]. In agreement with this, a randomized cross-over study in healthy humans found that a great portion of the ingested (−)- epicatechin and procyanidin B1 was metabolized by the colonic microbiota to produce phenyl-γ-valerolactones as the major microbial metabolites [60]. In this study, microbial degradation of larger procyanidins was substantially lower, possibly to the inhibition of digesting enzymes or to the antibacterial properties exhibited by these compounds. Other human studies analyzing the bioavailability of flavan-3-ols, reported high levels of phenyl-γ-valerolactones in the circulation and urinary excretion after ingestion of a red grape pomace drink [71] and apple juice [72]. In the colonocytes and hepatocytes, these microbial products undergo further metabolism by phase II enzymes to produce conjugated derivatives. Margalef et al. [73] analyzed the tissue distribution of metabolites derived from a grape-seed proanthocyanidin extract (GSPE) 2 h after ingestion by rats. These authors detected a few microbial metabolites (methyl conjugated phenols) at low concentrations in the colon tissue, while most phase II metabolites (glucuronidated and methyl-glucuronidated forms) were found in the kidneys and liver. In humans, the major contributors to the excretion of phenyl-γ-valerolactones after ingestion of a red grape pomace drink, are sulfated and glucuronidated conjugates of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone [71].

5. In vitro, in vivo and ex vivo studies on the benefits of PACs for intestinal dysfunction

During the last decade, the beneficial properties of PACs for intestinal function have been reported in several studies performed with cell-culture models and experimental animals (**Tables 1** and **2**). This experimental data indicate that PACs contribute to maintaining the intestinal barrier and improving mucosal inflammation induced by environmental insults. However, there are few studies on the effect of PACs on human intestinal health, although epidemiological studies connect PAC-rich food consumption with a lower risk of colorectal cancer [88].

Extract or compound	Concentration	Time of incubation	Experimental model	Permeability and/or inflammatory inductor	Outcomes		Ref.
					Permeability/integrity	Inflammation/oxidative stress	
Apple procyanidins	13-50 μg mL^{-1}	6 h	Caco-2	PMA (300 ng mL^{-1})	ND	↓ IL-8 release	[82]
Apple procyanidin dimer fraction	50-150 μg mL^{-1}	24 h	Caco-2	LPS (50 μg mL^{-1})	↑ Occludin. ↑ ZO-1.	↓ NF-κB and TNF-α gene expression. ↑GPx, SOD, HO-1.	[10]
Cranberry procyanidins	250 μg mL^{-1}	Preincubation for 24 h	Caco-2/15 cells	Fe/Asc mixture (200 μM/2 mM) or LPS (200 μg mL^{-1}) for 6 h	ND	↓ PGE_2 accretion. ↓ COX-2 protein content. ↓ TNF-α and IL-6 protein content.	[83]
Hexameric procyanidins	20 μM	Preincubation for 30 min	Caco-2	TNF-α (10 ng mL^{-1}) for 60 min	ND	↓ IκBα phosphorylation. ↓ NF-κB p50 and RelA nuclear translocation. ↓ NF-κB-DNA binding. ↓ iNOS mRNA and protein content ↓ ROS.	[84]
Nut polymeric-PAC fraction	4.8-12 (mg cyanidin equivalents mL^{-1})	Preincubation for 1 h followed by co-incubation for 24 h with the inflammation inductor	Caco-2	IL-1β (25 ng mL^{-1})	↑ TEER. ↓ FSA permeation.	↓ IL-6 and IL-8 release. ↓ IκBα phosphorylation. ↓ RelA nuclear translocation.	[85]
Cocoa procyanidin polymers	100 μg mL^{-1}	Preincubation for 24 h	Caco-2	DSS (2% w v^{-1}) for 48 h	ND	↓ IL-8 release.	[86]
			HT-29	TNF-α (5 ng mL^{-1}) for 6 h	↓ FD (4 kD) permeation	ND	
Procyanidin B2	50 μM	Preincubation for 24 h, co-incubation with the inflammation inductor for a further 48h	Caco-2/HT29-MTX co-culture	LPS-activated Raw264.7 medium	≈ TEER. ↑ Claudin-7. ↑ Occludin. ↓ ZO-1.	ND	[76]
Various PAC-rich extracts (apple and avocado peel, cranberry and grape)	12.5-50 μg mL^{-1}	24 h	Caco-2	*p*-Cresol (3.2 mM)	↑ TEER. ↓ FD (4 kD) permeation.	ND	[87]

FSA, fluorescein-5-(and-6)-sulfonic acid trisodium salt. ND, not determined.

Table 1.
Interaction of PACs with intestinal permeability markers in cell culture and animal models.

Extract or compound	Dose (way of administration)	Time of administration	Experimental model	Permeability and/or inflammatory inductor	Outcomes		Ref.
					Permeability/integrity	Inflammation/oxidative stress	
GSPE	5, 25 or 50 mg kg^{-1} bw (daily oral gavage)	3 weeks (after 15 weeks of cafeteria diet)	Diet-induced obese Wistar rat	Long-term cafeteria diet (18 weeks)	*Ileum:* ↑ ZO-1 gene expression.	*Ileum:* ↓ IL-1β gene expression. ↓ iNOS gene expression. ↓ MPO activity. ↓ ROS.	[14]
GSPE	500 mg kg^{-1} bw (daily oral gavage)	17 weeks every other week or 10 days (before cafeteria diet).	Diet-induced obese Wistar rat	Long-term cafeteria diet (17 weeks)	↓ Plasma OVA *Duodenum, ileum and colon:* ↑ TEER (*ex vivo*). *Ileum:* ↑ Claudin-1 gene expression.	*Ileum:* ↓ MPO activity.	[8]
GSPE	100 or 500 mg kg^{-1} bw (daily oral gavage)	2 weeks (after 15 weeks of cafeteria diet)	Diet-induced obese Wistar rat	Long-term cafeteria diet (17 weeks)	↓ Plasma OVA *Ileum and colon:* ↑ TEER (*ex vivo*). *Ileum:* ↑ Claudin-1 gene expression.	*Duodenum and colon:* ↓ TNF-α release (*ex vivo*). Ileum: ↓ MPO activity.	[15]
Pyracantha fortuneana fruit PAC-rich extract	0.4 or 1 g 100 g^{-1} of dry feed weight (orally)	8 weeks (after second week of high-fat diet).	Diet-induced obese Sprague Dawley rat	High-fat diet (10 weeks)	↓ LMR. ↑ Occludin (segment not specified). ↑ ZO-1 (jejunum).	ND	[88]
GSPE	100, 200 or 400 mg kg^{-1} bw (daily oral gavage)	7 days (after second TNBS-induced colitis)	Wistar rat with TNBS-induced recurrent ulcerative colitis	TNBS (ir. injection of 80 mg kg^{-1}, 30 mg kg^{-1} after 16 days)	ND	*Colon:* ↓ TNF-α. ↓ MPO and iNOS activities. ↓ IKKα/β and IκBα phosphorylation. ↓ NF-κB nuclear translocation. ↓ MDA. ↑ GPx and SOD activities.	[89,90]
GSPE	100, 200 or 400 mg kg^{-1} bw (daily oral gavage)	7 days (after TNBS-induced colitis)	Wistar rat with TNBS-induced ulcerative colitis	TNBS (ir. injection of 100 mg kg^{-1})	ND	*Colon:* ↓ IL-1β. ↓ MPO activity. ↓ IKK activity. ↓ IκBα phosphorylation. ↓ RelA protein content.	[91]

Extract or compound	Dose (way of administration)	Time of administration	Experimental model	Permeability and/or inflammatory inductor	Outcomes		Ref.
					Permeability/integrity	Inflammation/oxidative stress	
Procyanidin B2	10, 20 or 40 mg kg^{-1} (daily oral gavage)	11 days	C57BL/6 mouse with DSS-induced colitis	DSS (2.5 g 100 mL^{-1} of drinking water for 9 days)	ND	*Colon:* ↓ MMP9. ↓ Cleaved caspase-1. ↓ RelA phosphorylation. ↓ TNF-α, IL-1β and IL-6 gene expression.	[92]
GSPE	1 g 100 g^{-1} of dry feed weight (orally)	16 weeks	IL10-deficient mouse prone to colitis	None (spontaneous colitis)	ND	*Colon:* ↓ TNF-α, IL-1β, IL-6 and IFN-γ gene expressions. ↓ MPO protein content and gene expression. ↓ RelA phosphorylation.	[93]
GSPE	0.1 g 100 mL^{-1} of drinking water (orally)	12 weeks	IL10-deficient mouse prone to colitis	None (spontaneous colitis)	ND	*Jejunum:* ↓ TNF-α and IFN-γ. ↑ IκBα protein content. ↑ iNOS gene expression.	[94]
GSPE	75 or 375 mg kg^{-1} bw (daily oral gavage)	15 days (before LPS administration)	Wistar rat with LPS-induced intestinal dysfunction	LPS (ip. injection of 0.3 mg kg^{-1})	↓ Plasma OVA *Duodenum:* ↑ JAM-A gene expression. *Ileum:* ↓ ZO-1, occludin, claudin-2, and JAM-A gene expressions.	*Duodenum:* ↓ COX-2 activity. *Duodenum and ileum:* ↓ MPO activity. Colon: ↓ ROS.	[13]

Bw, body weight. LMR, lactulose to mannitol ratio. Ir., intrarectal. Ip., intraperitoneal. ND, not determined.

Table 2.
Interaction of PACs with permeability and inflammatory markers in animal models of intestinal dysfunction.

In vitro models of inflammation have been fundamental in the comprehension of cellular mechanisms driving physiological effects of bioactive molecules. Studies on intestinal dysfunction have employed human colon carcinoma cell lines, being Caco-2 the most well-established and widely used model of the human intestine barrier ([89] and **Table 1**). Mucus producer [79], macrophages [90], and B cell lines [91] have been employed in co-culture systems to explore the interaction between cell populations. Although there is a strong trend in the industry toward replacing animal experiments with human cell-culture based models [92, 93], there are no in vitro models of the human intestine that replicate the complex interplay between cell types and the regulation of the barrier function by the mucosal innate and adaptive immunity. Therefore, most physiologically relevant data on intestinal dysfunction comes from the animal model. Most in vivo studies testing the effect of PAC supplementation on intestinal health have been performed in diet-induced obesity models and chemical-induced colitis models. The first resemble intestinal alterations seen in humans with metabolic syndrome [43]. The latter closely mimic histopathological features of human colitis and are frequently used to study the pathophysiology of IBD and the effectiveness of novel therapeutic drugs [94]. Notably, PAC-rich grape-seed extracts (GSPE) are among the most studied botanical extracts, mainly by in vivo approaches in rodents (**Table 2**).

5.1 In vitro studies of barrier integrity

The data available on the interaction between PACs and permeability and inflammation markers in cell models of intestinal dysfunction are summarized in **Table 1**. Caco-2-based models have shown to be responsive to pro-inflammatory stimulation, producing a wide range of inflammatory mediators and increasing the paracellular permeability. Pro-inflammatory agents such as LPS, phorbol 12-myristate 13-acetate (PMA), and cytokines (TNF-α and IL-1β) have been used in multiple studies testing the effect of PAC molecules and PAC-rich botanical extracts on Caco-2 cells [10, 74, 77, 78]. Stimulated-Caco-2 cell monolayers incubated with PACs generally show a reduction in gene expression and secretion of TNFα, IL-6, and IL-8 [10, 74, 75, 77], which is often linked to the downregulation of NF-κB signaling at different levels [10, 76, 77]. An increased expression of antioxidant enzymes, such as glutathione peroxidase (GPx), superoxidase dismutase (SOD), and hemeoxygenase 1 (HO-1), has also been reported [10].

When permeable support systems such as transwell or Ussing chamber (UCh) are used, alterations in barrier integrity and paracellular permeability of epithelial cell monolayers are evaluated by transepithelial electrical resistance (TEER), an electrophysiological parameter that measures ion conductance across the monolayer, and by the transepithelial transport of molecular markers such as Lucifer yellow (LY) and fluorescently labeled dextrans (FD) [95, 96]. Some in vitro studies have associated PACs with increased TEER and decreased transport of permeability markers in the context of barrier dysfunction [77, 78, 80]. The expression levels of TJ proteins (claudins, occludins, and ZOs) often correlate, but not always [79], with intestinal permeability and are also considered markers of epithelial integrity. Bitzer et al. [78] found that the dextran sodium sulfate (DSS)-induced loss of barrier function in Caco-2 cells was significantly inhibited by polymeric PACs of cocoa but not by oligomers. Moreover, a higher barrier-protective activity was determined in PACs with DP ≥ 7, which were able to reduce the detrimental effect of DSS in a dose-dependent fashion [78]. Effectiveness of procyanidin B2 ameliorating dextran sodium sulfate (DSS)-induced permeability alterations was examined using a Caco-2/HT29-MTX co-culture model [79]. Although procyanidin B2-incubated cells showed increased levels of the TJ proteins claudin-7, occludin, and ZO-1, these

changes did not reduce TEER loss. Altogether, these results suggest that the ability of PACs to strengthen the intestinal barrier integrity depends on the degree of polymerization (DP).

5.2 In vivo studies of diet-induced intestinal permeability

The cafeteria (CAF) diet is a self-selected high-saturated fat/high-refined sugar diet that stimulates hyperphagia and a rapid weight gain in experimental animals [97, 98]. In this feeding regime, highly palatable and energy dense foods commercially available, such as muffins, biscuits, bacon, sausages, and sugared milk, are offered *ad libitum* [15, 99]. A long-term CAF diet (62% carbohydrate (mostly sugar), 23% lipid, and 13% protein) has negative effects on intestinal function in rodents, increasing intestinal permeability, and inducing mucosal inflammation [43]. We have described the beneficial effects of administering GSPE against the intestinal dysfunction induced by a long-term CAF diet (17–18 weeks) in Wistar rats [8, 14, 15]. The composition of the GSPE used in these studies has been analyzed in detail [100]. Both nutritional (5–50 mg kg^{-1} [14]) and pharmacological (100–500 mg kg^{-1} [8, 15]) doses of GSPE administered orally as a preventive [8] or counteractive treatment [14, 15], tended to reduce intestinal inflammatory markers such as TNF-α release or myeloperoxidase (MPO) activity (an indicator of neutrophil infiltration in tissues). The reduction of plasma ovalbumin (OVA), an in vivo marker of intestinal permeability, was supported by (1) the increase in TEER in small and large intestine segments. This parameter is determined *ex vivo* by UCh-based protocols [8, 15]; and (2) by the upregulation of TJ proteins such as ZO-1 [14] and claudin-1 [8, 15]. Notably, the protective effect of GSPE in the intestinal barrier function was linked to the amelioration of metabolic endotoxemia (reduction of plasma LPS) and systemic inflammation (reduction of plasma TNF-α) in obese rats [15, 101]. Other authors have also reported the upregulation of ZO-1 and claudin-1 TJ proteins in high-fat fed rats supplemented with other PAC-rich extracts [81].

5.3 In vivo studies of chemical-induced intestinal dysfunction

Chemical agents administered orally to induce colitis in rodents include trinitrobenzene sulfonic acid (TNBS) and DSS. These agents erode the colonic mucosal lining and produce the loss of the intestinal barrier function and colonic inflammation. In these models, the severity of outcomes depends on the dose of the chemical agent and the frequency of administration. Li et al. [102] found that intragastric administration of GSPE in rats at pharmacological doses (100–400 mg kg^{-1} d^{-1}) prior to TNBS-induced recurrent colitis, reduced weight loss, and attenuated macro- and microscopic tissue damage scores in the colon. This protective effect was accompanied by a reduction in oxidative stress (malondialdehyde; MDA), inflammation (IL-1β), and neutrophil infiltration (MPO activity) in colonic tissues. Remarkably, the beneficial effects of low to high doses of GSPE were comparable to those of sulfasalazine (200 mg kg^{-1} d^{-1}), a potent inhibitor of NF-κB. Subsequent studies carried out by these authors with the same model, confirmed the role of the GSPE down-regulating NF-κB response [83, 84]. A preventive effect of procyanidin B2 was also evidenced in a mouse model of DSS-induced colitis [85]. Administration of procyanidin B2 (10–40 mg kg^{-1} d^{-1}) attenuated the severity of tissue damage in the colon and reduced the levels of matrix metalloproteinase-9 (MMP-9), a marker of macrophage infiltration. In addition, inhibition of the NF-κB signaling and of NLRP3 inflammasome activation was observed, with a concomitant reduction in the gene expression of pro-inflammatory cytokines. Overall, the benefits of procyanidin B2 administration, especially at the

highest dosage (40 mg kg^{-1}), were comparable to those of mesalazine (200 mg kg^{-1}), a COX inhibitor. The authors suggest that these effects were largely due to the reduction in activated macrophages infiltrating colonic tissues, probably driven by ROS clearance.

5.4 Other in vivo studies with animal models

The IL-10 deficient mouse is a classic knockout model that develops spontaneous colitis under pathogen-free conditions. Some authors have explored the influence of GSPE in this model, supplementing colitic animals with 0.1–1 g 100 g^{-1} of dry feed weight for 12–16 days [86, 87]. These studies evidenced a reduction of multiple inflammatory markers in the jejunum and colon, such as TNF-α, IL-1β, IL-6, and IFN-γ gene expressions, as well as MPO activity. This anti-inflammatory effect was associated with the inhibition of the NF-κB signaling. Interestingly, GSPE supplementation also increased the density of goblet cells in the jejunum of treated animals, suggesting that there is an alternative mechanism by which inflammation is attenuated.

Cardoso et al. [13] recently tested both dietary (75 mg kg^{-1}) and pharmacological doses of GSPE (375 mg kg^{-1}) in a rat model of mild intestinal dysfunction induced by intraperitoneal injection of LPS. GSPE was administered daily by oral gavage for 15 days prior to LPS-induced intestinal dysfunction. LPS enhanced intestinal permeability and induced both oxidative stress and inflammation. GSPE-treated animals reduced OVA permeation to the circulation, MPO activity and COX-2 in the small intestine tissues, and reactive oxygen species (ROS) levels in the colon. Furthermore, a gene expression analysis with a low-density microarray technique revealed that unlike the dietary dose of GSPE, the pharmacological dose had a striking effect on the LPS-gene expression profile, showing a strong modulation of multiple genes associated with chemokines and ILs, including upregulation of the anti-inflammatory cytokine IL-13.

5.5 Human ex vivo studies

Although the use of animal models is the predominate approximation at preclinical stages for testing novel therapies in intestinal permeability, there is a strong trend in the industry towards replacing animal experiments with human cell-culture based models [92, 93]. Nevertheless, advantages related to the usefulness of in vitro models for screening of bioactives and exploring action mechanisms, are offset by limitations regarding the mimicking of the in vivo situation and translation to the human [103]. Thus, some human ex vivo models have been proposed to test immunomodulatory properties of drug candidates in intestinal explants from IBD patients [104, 105]. Intestinal function can also be studied with UCh-based protocols. The UCh system consists of two halves with an opening between them, where mucosal tissue is adapted, thus isolating the apical and basolateral sides of the tissue. This technique has been applied for studying drug absorption [106] and secretion of enterohormones [107] in human endoscopic biopsies. An advantage of UCh models over explant-based models is that UCh models make it possible to measure the electrophysiological parameters, including TEER [106]. All these set-ups permit analyzing the cytokine profiling of intestinal explants or biopsies retaining their in situ conditioning in a polarized fashion [105, 108]. We have employed the UCh to determine TEER and cytokine release (TNF-α) in intestinal tissues from cafeteria diet-induced obese rats treated with GSPE [8, 15]. It could also be useful for testing the effect of bioactives on dysfunctional human intestine.

A feature of ex vivo models is that screening of drug effects does not compromise the patients by exposing them to unknown outcomes.

5.6 Clinical trials

Translation of doses of PAC-rich extracts used in rodent models of intestinal dysfunction to human equivalent doses (HED) indicates that pharmacological doses (up to 5 g d^{-1} for a 60 kg person) could be required to achieve beneficial effects in clinical trials [14, 15]. Thus, the first uncertainty involved in assessing the use of PACs as therapy agents in humans, is safety. Grape seed and skin proanthocyanidin-rich extracts have been subjected to toxicological tests in rats to determine their safety for use in functional foods [109–111]. In these studies, the median lethal dose (LD50) was found to be greater than 5000 mg kg^{-1} bw (HED of ≈50 g) when administered once by oral gavage, and 1400–2000 mg kg^{-1} d^{-1} (HED of ≈14–20 g d^{-1}) was found to be the no-observed-adverse-effect level (NOAEL) for systemic toxicity in sub-chronic administration. A recent study evaluated the safety and tolerability of GSPE intake (up to 2.5 g d^{-1}) in a small number of healthy adults for a 4-week period and found a good tolerability without adverse effects on hematological or biochemical parameters [47].

To date, there are few clinical studies that evaluate the influence of PACs on intestinal inflammatory conditions. A clinical study revealed that the postprandial increase of plasma LPS associated with the intake of a high-fat meal was significantly reduced in obese subjects who consumed 1 g of GSPE [112]. As translocation of LPS to the circulation is considered an indicator of intestinal permeability and a critical factor in the appearance of systemic low-grade inflammation in patients with metabolic syndrome [113], reduction of postprandial endotoxemia could be particularly interesting from a therapeutic perspective. Large double-blind clinical studies need to be conducted to provide more information on PAC clinical efficacy in intestinal dysfunction so that these phytochemicals can be used therapeutically to improve intestinal health in obese and IBD individuals.

6. Biochemical and molecular mechanisms underlying the barrier-protective and anti-inflammatory properties of PAC in the intestine

PACs were often considered to be nutritionally undesirable due to their ability to form complexes with macronutrients and reduce the activity of virtually any enzyme implicated in digestion [114, 115]. Nevertheless, based on the anti-cancerous, anti-mutagenic, and anti-microbial activities these phytochemicals elicited in laboratory experiments, a role in the modulation of the metabolism and immune system was suggested [115]. The ability of PACs to form cross-links with biomolecules can be attributed to the hydroxyl groups and aromatic rings in their structure that can establish hydrogen bonds and hydrophobic interactions [116]. PACs have a significant affinity for proline-rich proteins and peptides [117]. In general, binding to proteins seems to increase with the DP as larger PAC molecules have more potential binding sites for the associations with proline residues [117]. The interaction results in effects determined by the biological function of the target protein. Thus, PACs not only alter enzymatic activity, but they may also prevent ligand-receptor interactions and the binding of transcription factors to their specific sites in DNA. In addition, some PAC molecules can be adsorbed non-specifically onto biomembrane surfaces [118], affecting their physical characteristics, such as fluidity and density, and potentially altering membrane-dependent processes,

including protein receptor activity [119]. Altogether, these effects lead ultimately to the alteration of cell signaling pathways and the modulation of gene expression.

6.1 Modulation of TJ integrity

The precise mechanisms underlying the improvement in intestine paracellular permeability due to PACs in inflammation are not yet completely elucidated; however, it is known that they lead ultimately to the upregulation (e.g., ZO-1 and claudin-1 [8, 13]) or downregulation (e.g., claudin-2 [86]) of TJ protein expression. Loss of TJ integrity in the pro-inflammatory state is mediated by the NF-κB signaling pathway and by the activation of protein kinases MAPKs, PI3Ks, AMPK, and MLCK [120]. MLCK is particularly crucial in actomyosin-based cytoskeletal functions and multiple studies highlight its important role in intestinal TJ remodeling [121, 122]. PACs reduce the production of pro-inflammatory mediators (e.g., TNF-α) and reactive oxygen species (i.e., iNOS activity) associated with enhancing intestinal permeability by antagonizing the NF-κB signaling pathway. In addition, PACs are potent inhibitors of kinases including MLCK [120, 123]. Contreras et al. [124] also suggested that there is an upstream novel mechanism associated with flavan-3-ols that leads to the prevention of TNF-α-induced intestinal permeability. In this study, TNF-α-stimulated Caco-2 monolayers incubated with (−)-epicatechin showed a reduction of NOX activity, an enzyme that also facilitates activation of TNF-α signaling. This effect was directly associated with the inhibition of ERK1/2 MAPK activity of IκB phosphorylation and of MLCK activation.

6.2 Interaction with bacterial endotoxins

Delehanty et al. [125] demonstrated that naturally occurring A- and B-type cranberry PACs were able to bind the lipid A moiety of LPS, exhibiting an affinity similar to that of polymyxin B, a potent LPS-binding molecule. In this study, PACs efficiently blocked endocytosis of bacterial LPS in a dose-dependent manner in HEK 293 (human embryonic kidney cells) that expressed receptors TLR4/MD-2 and CD14, thus preventing the induction of the NF-κB signaling pathway without any interaction with cellular components. However, other authors reported that PACs isolated from cocoa beans did not abrogate the binding of LPS to TLR4 in cultivated human dendritic cells [126]. PAC-LPS binding has been linked to the reduction of the post prandial increase in blood LPS associated with the ingestion of a high-fat meal in obese subjects ingesting an oral dose of GSPE [112].

7. PACs modulation of intestinal microbiota

Diet plays an important role in the composition of intestine microbiota, promoting or inhibiting growth of microorganisms [127]. Alterations in the composition and metabolism of the intestinal microbiota (dysbiosis) have also been associated with the consumption of high-saturated fat diets in rodents and humans [128, 129]. In fact, metagenomic analysis of the intestinal microbiome in Western populations has shown a reduction not only of microbial diversity, but also of functional potential [130]. Dysbiosis is linked to obesity-associated intestinal inflammation, although the "egg or hen" question related to the cause-effect relationship is not answered yet [131]. High-fat intake in rodents often decreases overall diversity of microbiota and the abundance of Bacteroidetes, and increases the relative abundance of Firmicutes [132, 133]. Several human studies have described similar associations [134, 135], but the importance of the ratio Firmicutes to Bacteroidetes remains controversial [136, 137], and some authors state that the

experimental results are not sufficiently consistent [138]. Interestingly, the existence of a colitogenic microbiota was demonstrated in T-bet−/− × RAG2−/− deficient mice whose spontaneous ulcerative colitis was horizontally transmissible to wild-type individuals when co-housed [139]. Although mechanisms by which dysbiosis trigger intestinal dysfunction are not fully understood, it is known that they involve the loss of immune tolerance due to local immune homeostasis disruption and continuous abnormal activation of TLRs [140].

Several authors have suggested that both dietary PACs, which are the substrates of intestinal bacteria, and the metabolites produced during PACs degradation in the colon may modulate and induce oscillations in the composition of the microbiota populations by means of prebiotic and antimicrobial effects against gut pathogenic microorganisms [141–144]. Dietary PACs, specifically longer polymers, reach the distal intestine nearly intact, where they become fermentable substrates for the commensal microbiota [145]. PACs have been associated with prebiotic properties, boosting the composition of several kinds of probiotics such as *Bifidobacterium* spp., *Lactobacillus* spp. [146] and the stimulator of mucus production *Akkermansia muciniphila* [147, 148]. Nevertheless, current evidence is somewhat controversial as effects described in different in vivo studies mainly performed with rodents, do not always agree. This suggests that interactions between PACs and microbiota depend largely on the botanical source, the types of molecules present in the extracts tested and the animal model [149].

A recent study by Casanova-Marti et al. [150] found that oral administration of GSPE in Wistar rats for 8 days resulted in profound changes in the cecal microbiota composition, reducing diversity indices and the ratio of *Firmicutes* to *Bacteroidetes*. Similar results were found in diet-induced obese Sprague Dawley rats supplemented with a PAC-rich extract of the *Pyracantha fortuneana* fruit, although in this study an increase in microbiota diversity was also reported [81]. GSPE supplementation in IL-10 deficient mice resulted in an increased abundance of *Bacteroides* and *Lactobacilli* [86]. Xing et al. [148] reported that the administration of procyanidin B2 in rabbits feeding a high-fat-cholesterol diet, promoted an increase in the relative abundance of *Akkermansia*. These authors proposed that the reduction of metabolic endotoxemia found in animals treated with procyanidin B2 was attributed to the ability of *Akkermansia* to retain the thickness of the intestinal mucus layer, thus reducing intestinal permeability and the leakage of LPS into the circulation [151].

Cueva et al. [146] found that in vitro fermentation of grape-seed monomers and PACs in human feces resulted in a reduced abundance of *Clostridium histolyticum*. Inhibition of the growth of some infectious microorganisms, such as the mentioned *C. histolyticum* in the intestine and *Helicobacter pylori* in the stomach [152], may be related to the anti-adherence activity that PACs have demonstrated in in vitro studies [153], as adherence to the epithelium is a prerequisite for colonization and infection of the intestinal gastrointestinal mucosa.

Finally, phenolic acids and phenyl-γ-valerolactones resulting from the colonic fermentation of PACs also exhibit a significant bioactivity in cell models and experimental animals [154]. They therefore may partially account for the beneficial anti-inflammatory effects reported in intestinal and peripheral tissues in vivo. Further research is needed to clarify the importance of these microbial products in health-promoting properties associated with the intake of PACs.

8. Conclusions and future perspectives

The health-promoting properties of PACs in the intestine are attributed not only to the antioxidant activity inherent to phenolic compounds, but also to the capacity

of these phytochemicals to interact with multiple biomolecules, including proteins, biomembrane lipids, and endotoxins. Bioactivity of PACs is highly structure-dependent and enriched botanical extracts composed by a large variety of molecular structures exert a wide range of unrelated physiological effects. In this way, PAC-rich extracts can modulate kinase activity, several signal transduction pathways implicated in the inflammatory response and the remodeling of TJs. Flavan-3-ol monomers and short PAC oligomers are absorbed by enterocytes and immune cells and exert a direct action on kinases and transcription factors. Bioactivity of larger oligomers and polymeric PACs do not require direct intestinal absorption and are able to bind protein receptors on the enterocyte and immune cell surfaces as well as luminal bacterial endotoxins, thus inhibiting pro-inflammatory signaling and improving barrier integrity. Due to the negligible absorption of large PAC molecules in the small intestine, phenyl-γ-valerolactones and phenolic acids produced by the microbiota metabolism in the colon are thought to play an important role in these health-promoting effects, and thus need to be further researched.

The barrier-protective properties of PACs are emerging as a potential adjunctive support to current therapies for managing obesity related intestinal dysfunction and IBD. However, there have been no large, well-designed clinical trials establishing the efficacy of these phytochemicals in chronic conditions. At preclinical stages, the use of animal models is the predominant approach for testing novel therapies for intestinal dysfunction, although several strategies for replacing animal experiments have been proposed. As there are still no studies on the impact of PACs on human intestinal health, ex vivo models of the human intestine could be a more physiologically reliable alternative to human cell lines and an alternative to animal experimentation in preclinical development.

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Conflicts of interest

The authors declare no conflict of interest.

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