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1 **Toxicity of polycaprolactone nanoplastics, pristine or weathered in environmental conditions, to**  
2 **human intestinal epithelial cells, *in vitro***

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14

15 **Abstract**

16 Plastic, used in almost all everyday products, is a major source of pollution, particularly in the form of  
17 micro- and nanoplastics (MNPs). MNP impact on health, particularly on the digestive tract, is still  
18 poorly understood, especially in vulnerable populations such as those suffering from inflammatory  
19 bowel disease. The aim of this study was to assess the *in vitro* toxicity of nanoplastics (NPLs) from a  
20 biodegradable polymer, polycaprolactone (PCL), both in its pristine state and after accelerated  
21 weathering in environmental conditions, the latter resulting in the release of potentially toxic PCL  
22 oligomers. To do so, we used *in vitro* models of genetic susceptibility to Crohn's disease (CD),  
23 consisting in co-cultures of HT29-MTX cells with Caco-2 cells engineered to express either wild-type  
24 (WT) or mutated *nucleotide-binding oligomerization domain 2* (NOD2), representative of healthy  
25 individuals (Caco-2 NOD2<sup>WT</sup>) or individuals with susceptibility to CD (Caco-2 NOD2<sup>1007fs</sup>).  
26 Physicochemical transformation of PCL NPLs upon weathering were characterized. Cells were  
27 exposed to pristine and aged PCL NPLs and their cytotoxicity, genotoxicity, inflammatory potential,  
28 impact on the cells' redox balance and unfolded protein response, as well as on the epithelial barrier  
29 integrity were evaluated. Results show that accelerated weathering increases the crystallinity and  
30 leads to fusion of PCL NPLs. PCL NPLs accumulate inside cells where they degrade and release some  
31 PCL oligomers. However, in the tested conditions, PCL particles, both pristine and aged, do not show  
32 any overt toxicity in both cell systems, irrespective of particle size and weathering status. These data  
33 confirm that PCL NPLs, may they be intact or partially dissolved, show only mild toxicity to colon cells,  
34 upon acute, short-term exposure of Caco-2 / HT29-MTX cells.

35

36 **Keywords:** Micro and nanoplastics; physicochemical characterization; environmental aging; toxicity;  
37 intestinal models; inflammatory bowel disease; polycaprolactone

38

## 1. Introduction

Plastic is an essential material, widely used in many sectors due to its advantageous properties and low cost [1]. Its massive production, which exceeded 400 million tonnes (Mt) in 2022, is causing major environmental pollution due to insufficient waste management, with only 10% of plastics being recycled [2, 3]. It is estimated that between 4.8 and 12.7 Mt of plastic waste enter the oceans every year [4]. This situation is exacerbated by plastics' resistance to natural degradation, leading to their accumulation and persistence in the environment for decades to centuries. Once in the environment, exposure of plastic waste undergoes photochemical, mechanical, hydrolytic, thermal and biological stress, which result in the formation of micro- and nanoplastics (MNPs) [5]. Microplastics (MPs) refer to particles smaller than 5 mm, while nanoplastics (NPLs) are generally defined as less than 1  $\mu\text{m}$ , although size definitions vary between studies [6, 7].

Human exposure to MNPs occurs mainly through ingestion or inhalation [8]. The presence of MNPs has been widely reported in drinking water and various food products, including seafood, salt, fish, crustaceans, as well as sugar, honey, and beer [9-11]. Assessing human exposure remains challenging due to the diversity of MNPs and dietary habits. However, a study by Senatirajah et al. estimates that individuals ingest between 0.1 and 5 g of MNPs per week [12], although it is currently recognized as being an overestimation. While a significant proportion of these MNPs is excreted and detected in faeces [13-16], some MNPs can cross biological barriers, reach the bloodstream and some of them have been identified in several human organs as reviewed recently [17]. To date, the health effects of MNPs remain poorly understood, particularly their impact on the digestive tract. In both vertebrates and invertebrates, ingestion of MNPs has been linked to intestinal barrier dysfunction, inflammation and dysbiosis, leading to symptoms similar to those observed in inflammatory bowel diseases (IBD). In zebrafish, exposure to polypropylene (PP) and polystyrene (PS) MNPs induces mucosal damage, increases intestinal permeability, causes dysbiosis and inflammation [18]. In mice, PS MNPs also accumulate in intestinal tissues, disrupting the gut barrier and triggering adverse effects in the intestine and liver, i.e., oxidative stress, inflammation and metabolic disorders, some of these effects being triggered by microbiota dysbiosis [19-21]. *In vitro*, most studies related to MNP toxicity were conducted with PS particles, showing low toxicity except at unrealistically high concentrations, while they heavily accumulate inside intestinal cells [22].

The impact of MNPs on vulnerable populations, particularly individuals with CD, which is one of the most prevalent IBD, remains largely unexplored. While the exact causes of CD are still unclear, the combination of genetic factors, environmental factors and immune system dysfunction has been proposed as playing a role in its development and aggravation, leading to intestinal barrier dysfunction

73 and abnormal immune responses in the intestine [23]. Yan et al. found a positive correlation between  
74 the presence of MNPs in stool of IBD patients and disease severity [15]. Additionally, exposure to PS  
75 MNPs exacerbates colitis symptoms in mice with DSS-induced colitis compared to healthy mice [24,  
76 25].

77 Given the reported adverse effects on health and the environment of non-biodegradable plastics, the  
78 development of biodegradable plastics is seen as an attractive alternative due to a shorter half-life in  
79 the environment leading to lower exposure [26]. Among these, polycaprolactone (PCL) stands out as a  
80 promising biodegradable polyester due to its ability to rapidly decompose through the action of  
81 microorganisms or through chemical hydrolysis in various environments [27]. The use of PCL is  
82 expanding, particularly for medical and biomedical applications (e.g., resorbable devices and drug-  
83 delivery systems) but also for applications such as food packaging, adhesives, agriculture, 3D printing  
84 and coatings (for review, see [27]). Although PCL is recognized by the FDA as non-toxic, its prolonged  
85 degradation time in the body raises concern about the potential release of oligomers, and about  
86 possible toxic effects of both PCL MNPs and oligomers, as some studies have reported that PCL MNPs  
87 cause perturbation of epithelial and immune cell normal function [28-30]. Such combination of MNPs  
88 and oligomers may be one of the environmental factors contributing to the onset or aggravation of  
89 CD, and up to now, although considered as important, the associated toxicity has been overlooked [31,  
90 32].

91 This work aimed at assessing the toxicological impact of PCL MNPs, either pristine or as a mixture of  
92 MNPs and their degradation products, as produced when exposed to environmental conditions,  
93 focusing on particles in the sub-micron size range. We previously characterized oligomer release from  
94 PCL NPLs upon artificial weathering [33, 34], showing that oligomers ranging from the mono to the  
95 hexamer were produced as PCL hydrolyses [33, 34]. Therefore, the first objective of this study was to  
96 determine whether weathering-driven changes in particle physicochemical properties, including  
97 oligomer release, may contribute to their toxicity. Second, this study aimed at evaluating their impact  
98 on the intestine, which was assessed on *in vitro* models of human intestinal epithelium representative  
99 of the colon, both healthy and with genetic susceptibility to CD. Traditional *in vitro* models used in  
100 toxicology studies related to IBD are models that replicate the active phase of the disease, by inducing  
101 inflammation through exposure of Caco-2 cells to strong inflammatory stimuli [35]. Here, we used an  
102 *in vitro* model of genetic susceptibility to CD, consisting in Caco-2 cells genetically modified to express  
103 either wild-type or mutated *nucleotide-binding oligomerization domain 2* (NOD2). NOD2 plays a crucial  
104 role in epithelial cell response to the microbiota and pathobionts by detecting muramyl dipeptide  
105 (MDP) from bacterial cell walls, which triggers NF- $\kappa$ B signalling and subsequent inflammatory response  
106 [36]. The NOD2 gene is associated with CD, with over 60 identified variants and 30% CD patients

107 showing one mutation in NOD2. The mutation that is most strongly linked to CD is the 1007fs mutation  
108 (NOD2<sup>1007fs</sup>) [37, 38], which results in a truncated protein that fails to recognize MDP, thus disrupting  
109 the NF-κB signalling [39]. Such cell models represent populations at risk of developing CD. Caco-2  
110 NOD2<sup>WT</sup> and Caco-2 NOD2<sup>1007fs</sup> cells were co-cultured with the mucus-secreting HT29-MTX intestinal  
111 cell line, to reproduce a mucus-secreting ileum-like epithelium [40, 41]. After characterizing the impact  
112 of weathering on PCL NPL physicochemical properties, the intracellular accumulation and impact of  
113 pristine and weathered PCL NPLs on cell viability, epithelial barrier integrity, ROS intracellular levels,  
114 DNA integrity and inflammatory response were assessed. This study provides valuable data related to  
115 the toxicological impact of the biodegradable MNPs, namely PCL NPLs and their degradation products,  
116 towards intestinal epithelial cells representative of populations at risk of developing CD.

117

## 118 **2. Materials and Methods**

### 119 **2.1. PCL particles**

120 PCL NPLs (with diameter 100 nm, 200 nm, 500 nm, or 1000 nm) were purchased from Phosphorex  
121 (Hopkinton, USA) as aqueous suspensions and stored at 4°C. These particles are aimed to be used for  
122 sustained drug delivery. All the physicochemical characterization and toxicity assays reported in this  
123 manuscript were obtained using these non-labelled PCL particles. For cell uptake experiments as  
124 assessed via confocal fluorescence microscopy, PCL NPLs were labelled with Disperse Blue 14 (DB14,  
125 1,4-Bis(methylamino)anthraquinone, ABCR #AB177338,  $\lambda_{ex.}/\lambda_{em.}$  640 nm/685 nm) using the solvent  
126 swelling method, leading to NPLs with unchanged size [42, 43]. To this end, a solution of DB14 at 10  
127 mg/mL was prepared in tetrahydrofuran (THF). One percent of this solution (v/v) and 10% THF were  
128 added to one volume of particle suspension, and the mixture was stirred on a rotary wheel at room  
129 temperature for 30 min. Two volumes of water were then added; then the particles were collected by  
130 centrifugation for 30 min at 15,000 x *g*, washed with water and centrifuged again. Finally, the pellet of  
131 DB14-labelled NPLs was resuspended in one volume of 20% ethanol.

### 132 **2.2. Accelerated weathering in a test chamber**

133 To simulate environmental degradation, PCL NPLs were aged in a Q-SUN Xe-1 test chamber (Qlabs)  
134 equipped with a xenon arc lamp that reproduces a light spectrum similar to sunlight. The aging  
135 conditions were set according to the ISO 4892-2:2013 guideline, which recommends an irradiance of  
136 1.44 W/m<sup>2</sup>/nm (as measured at 420 nm) and a temperature of 40°C. NPLs were weathered for 96 hours  
137 in this condition, under continuous agitation in hermetically sealed quartz cuvettes. A 200 µL aliquot  
138 of the suspensions was collected every 24 hours (i.e., at 24, 48, 72, and 96 h) to monitor degradation

139 over time, which has been reported previously [33, 34]. For the present study, we chose 96h as the  
140 degradation duration, as it maximized PCL degradation, while avoiding complete dissolution. The aged  
141 suspensions were stored at 4°C until further experiments.

## 142 **2.3. Physicochemical characterization**

### 143 **2.3.1. Transmission electron microscopy (TEM)**

144 Primary diameters of PCL NPLs were measured on transmission electron microscopy (TEM) images of  
145 100 particles, obtained using negative staining on a grid. PCL NPLs were deposited on the clean side of  
146 a carbon film on mica and stained with 2% uranyl acetate or 2% phosphotungstic acid. Then, samples  
147 were transferred onto a 400-mesh copper grid and imaged using a Tecnai 12 LaB6 electron microscope  
148 equipped with a Gatan Orius 1000 CCD camera, operating at an accelerating voltage of 120 kV and  
149 defocus values between 1.2 and 2.5  $\mu\text{m}$ .

### 150 **2.3.2. Dynamic light scattering (DLS)**

151 Size distributions and polydispersity indexes of 100  $\mu\text{g}/\text{mL}$  PCL NPLs suspensions in ultrapure water  
152 and in cell culture medium, immediately after dilution and after 24 h of incubation at 37°C, 5%  $\text{CO}_2$ , at  
153 the concentrations used for cell exposure, were assessed by DLS using a Litesizer™ 500 (Anton Paar,  
154 USA). Dilution in cell culture medium did not significantly change their size distribution, compared to  
155 dilution in water. Surface charge was estimated by measuring the zeta potential using a Univette in a  
156 Litesizer™ 500 (Anton Paar, USA). Data were collected and analyzed using the Kalliope™ software.

### 157 **2.3.3. FTIR and Raman Spectroscopy**

158 FTIR spectra were recorded on particle suspensions that were air-dried on foil, using a Nicolet iS50  
159 spectrometer (Thermo Scientific, USA) equipped with a diamond ATR module. A total of 32 scans were  
160 recorded with a resolution of 4  $\text{cm}^{-1}$  in the 4000 to 400  $\text{cm}^{-1}$  range. FTIR spectra were analyzed using  
161 the OMNIC software (Thermo Scientific, USA). Each spectrum represents the average of three  
162 measurements recorded on different regions of the sample. Raman spectra were obtained using a  
163 WITec alpha300 RA Raman spectrometer (Oxford Instruments, Germany). For this, particles were  
164 deposited on a calcium fluoride substrate (ESCO Optics) and spectra were acquired at 532 nm  
165 excitation wavelength with a spectral resolution of 0.4  $\text{cm}^{-1}$ , via a 100x objective (NA 0.9). The grating  
166 was 2400 lines/mm and the laser power was set 10 mW.

167

## 168 2.4. Cell culture and exposure

169 Caco-2 NOD2<sup>WT</sup> and Caco-2 NOD2<sup>1007fs</sup> cells were obtained by transduction of the Caco-2 cell line (ATCC  
170 HTB-37, passages 14-40) using a lentivirus expressing either the wild-type NOD2 gene (NOD2<sup>WT</sup>) or the  
171 NOD2 gene carrying the 1007fs mutation (NOD2<sup>1007fs</sup>). These cell lines were kindly provided by F.  
172 Barreau (INSERM U843, Toulouse) and their characterization has been described previously [44, 45].  
173 Mucus-secreting HT29-MTX cells were generously donated by Thecla Lesuffleur (INSERM, Paris) [46].  
174 Cells were seeded in 100 mm Petri dishes (Falcon®) and maintained at 37°C in a 5% CO<sub>2</sub> humidified  
175 atmosphere in Dulbecco's Modified Eagle Medium/GlutaMAX™ (DMEM, Gibco™) to which was added  
176 10% decompartmented foetal bovine serum (FBS) (Gibco™), 1% non-essential amino acids (MEM NEAA  
177 100X, Gibco™), 50 units/mL penicillin and 50 µg/mL streptomycin (Sigma-Aldrich™) (complete cell  
178 culture medium). Cells were passaged twice a week with Trypsin-EDTA. Co-cultures were established  
179 by seeding 90% of Caco-2 NOD2<sup>WT</sup> or Caco-2 NOD2<sup>1007fs</sup> cells with 10% of HT29-MTX cells, in multi-well  
180 plates at a density of 80,000 cells/cm<sup>2</sup>. They were grown either for 24 h or for 21 days post-confluence  
181 to mimic a non-differentiated epithelium or a mature epithelium with tight junctions and microvilli,  
182 respectively. Then, they were exposed for 24 h to PCL particles diluted in complete cell culture  
183 medium. The tested concentrations were those that are commonly used in toxicological studies,  
184 making possible the comparison of our results with those reported in the literature. The highest  
185 concentrations, ranging from 12.5 to 200 µg/mL, were used exclusively to assess the impact of the  
186 particles on cell viability. For all other assays, lower concentrations (12.5, 25, and 50 µg/mL) were used.  
187 Considering a human ingestion of up to 5 g of MNPs per week [12, 47], the human intestine surface  
188 being approximately 32 m<sup>2</sup> [48], it can be estimated that humans are exposed to 0.22 µg MNPs/cm<sup>2</sup> of  
189 intestine per day. We exposed cells to 12.5-50 µg/ml PCL in most experiments, i.e., 3.12-12.5 µg/cm<sup>2</sup>.  
190 Therefore, the *in vitro* exposure dose was 15 to 60 time higher than the estimated daily human  
191 exposure. This is high, but this can be considered as a worst-case scenario, given that the intestinal  
192 transit is not linear and therefore some areas of the intestine may be exposed locally to much higher  
193 doses of NPLs than others. Each condition was applied in at least three separate wells (technical  
194 replicates) and all tests were repeated independently at least three times with cells at different  
195 passage numbers (biological replicates).

196

## 197 2.5. Cell model characterizations

### 198 2.5.1. Mucus secretion

199 The presence of mucus was assessed using Alcian blue staining. Cells were seeded in six-well plates  
200 and grown for 21 days, changing the medium every 2-3 days until their differentiation into enterocyte-

201 like cells [41]. At the end of this differentiation period, cells were washed twice with PBS and fixed for  
202 20 minutes with a mix of 75% ethanol (90°) and 25% acetic acid (3%). Then, a 1% Alcian blue (Sigma-  
203 Aldrich, B8438) solution prepared in 3% acetic acid was added to the wells and incubated for 30  
204 minutes at room temperature (RT). Stained cells were observed using a phase contrast microscope  
205 (Axiovert 25, Zeiss).

#### 206 **2.5.2. Immunostaining**

207 For immunostaining, cells were seeded in culture chambers on glass slide (Nunc™ Lab-Tek™ II Chamber  
208 Slide™ System, Thermo Scientific™). After differentiation, cells were washed with PBS and fixed for 20  
209 min at RT with 4% paraformaldehyde (PFA), then permeabilised for 5 min with 0.2% Triton X-100 and  
210 washed three times with PBS. Non-specific proteins were blocked with 3% bovine serum albumin (BSA)  
211 for 1 h at RT, then cells were incubated overnight at 4°C with anti-ZO-1 (Sigma-Aldrich, AB2272) or  
212 anti-MUC5AC (ABclonal, A17325) primary antibodies. After three washes in 3% BSA, they were  
213 incubated with anti-rabbit-IgG Atto 550 antibody (Sigma-Aldrich, 43328) for 2 hours at RT, then washed  
214 three times with PBS. After labelling cell nucleus with Hoechst 33342 (Sigma-Aldrich, 14533) for 30  
215 min at RT, samples were washed three times with PBS and coverslips were mounted on slides with  
216 Prolong Gold antifade solution (ThermoFisher Scientific). Images were captured using an Olympus IX83  
217 inverted microscope fluorescence microscope and processed with the Fiji software.

#### 218 **2.6. Particle and oligomer cell uptake**

219 First, cellular internalisation of PCL particles was assessed by flow cytometry as previously described  
220 [28]. Cells were grown for 24 h in 12-well plates and exposed to 50 µg/mL DB14-labelled PCL particles  
221 (PCL-DB14) for 24 h. Then, cells were collected, washed twice with PBS and analyzed by flow cytometry  
222 (Melody, Becton Dickinson). Then, PCL particle intracellular accumulation and distribution were also  
223 imaged by TEM. Cells were exposed for 24 h to 50 µg/mL PCL particles, then washed twice with 0.1 M  
224 PHEM buffer and fixed for 1 h with 2% formaldehyde/0.2 % glutaraldehyde. They were post-fixed with  
225 1% osmium tetroxide to which 1% uranyl acetate was added. Samples were dehydrated with a graded  
226 series of ethanol and embedded in Epon resin. The samples were then cut into 1-2 mm strips and  
227 embedded a second time in Epon, in the perpendicular orientation, so as, after cutting them, sections  
228 allowing observation of the entire thickness of the cells were obtained, i.e., from the apical pole to the  
229 basolateral pole. These samples were cut as 80 nm ultrathin sections, which were observed using a  
230 JEOL 1200EX TEM operating at 80 kV.

231 For detection of PCL oligomers (oPCL) within cells, cells were grown in 12-well plates for 24 h, then  
232 they were exposed for 24 h or 48 h to 200 µg/mL oPCL standard or 200 nm PCL NPLs. Cells were washed  
233 with PBS and harvested using trypsin-EDTA. After washing with PBS, cells were lysed via two freeze-

234 thaw cycles. Lysates were centrifuged for 10 min at 10,000 x *g* in 0.2 µm filter units and filtrates were  
235 analyzed by HPLC-MS/MS as described previously [34]. Samples were injected into a ExionLC AD series  
236 chromatographic system (SCIEX, Framingham, MA) equipped with an Nucleodur C18 HiTec column (1.8  
237 µm particle size, 2×100 mm ID, Macherey Nagel, Gutenberg, France). The column oven temperature  
238 was set at 50°C and the flow rate was 0.35 ml min<sup>-1</sup>. The mobile phases were a 2 mM ammonium  
239 formate aqueous solution containing 0.1 % formic acid (A) and acetonitrile with 0.1 % formic acid (B).  
240 At 0 min, the composition of the gradient was pure A. The proportion of B was then increased to 10 %  
241 in 12 min, set to 60 % at 20 min and reached 100 % at 22 min. The latter composition was maintained  
242 for 3 min. At the outlet of the column, the flow was directed toward the turbo ion spray source of a  
243 6500+ Qtrap mass spectrometer (SCIEX) operated with the Analyst software (version 1.7, SCIEX). The  
244 source temperature was set at 550°C. The pressure of curtain gas, nebulization gas and heated sheath  
245 gas were set at 45, 60 and 60 psi respectively. The flow of exhaust gas (air) was set at 25 L min<sup>-1</sup>. The  
246 ion spray voltage was 4500 V. Quantitative analyses of oPCL in aged PCL NPL suspensions were  
247 performed in the single ion monitoring (SIM) detection mode, with the [M-H]<sup>-</sup> pseudo-molecular ion  
248 of each oligomer (monomer to heptamer), used as targeted ions. Quantification in cellular extracts  
249 was performed using the multiple reaction monitoring mode (MRM), which is more sensitive. For this  
250 purpose, signals corresponding to the fragmentation of targeted pseudo-molecular ions into PCL  
251 monomer and dimer were monitored. Quantitative data were inferred from external calibration  
252 curves, plotted from standards prepared as follows: pure 6-hydroxyhexanoic acid (6-OH-Hx, 30 mg,  
253 Thermo Scientific) was heated for 186 h at 50°C, then 1 mL of ultrapure water was added and the  
254 suspension was sonicated for 20 min before being diluted to a concentration of 1 mg/mL. Then, the  
255 solution was then filtered analyzed. It was found to contain oPCL carrying between 1 and 6 monomeric  
256 units. Their respective proportions were inferred from the total ion current in a HPLC-mass  
257 spectrometry analysis.

258

## 259 **2.7. Cell viability**

260 PCL particle impact on cell viability was assessed using the WST-1 assay (Roche). Cells were seeded  
261 into transparent 96-well plates. They were grown for 24 hours or 21 days after seeding to obtain either  
262 non-differentiated or differentiated co-cultures, respectively. Then, cells were exposed to PCL particles  
263 for 24 hours. PS-NH<sub>2</sub> 50 nm particles (Merck, #L0780) were used as a positive control at either 100  
264 µg/mL (non-differentiated co-cultures) or 500 µg/mL (differentiated co-cultures), since differentiated  
265 co-cultures are known to be more resistant to toxic substances due to organization into a tight barrier  
266 with microvilli. Therefore, it requires higher concentration of positive control substances to achieve

267 significant impact [49]. At the end of incubation, the exposure medium was replaced with 100  $\mu$ L of a  
268 10-fold diluted WST-1 solution prepared in FBS-free medium. After 1.5 hours of incubation,  
269 absorbance was measured at 450 nm and corrected for absorbance at 650 nm, using a Spectramax ID3  
270 (Molecular Devices). Results were normalized with respect to the absorbance of control (unexposed)  
271 cells.

272

## 273 **2.8. Intracellular reactive oxygen species (ROS) level**

274 ROS levels were quantified using the dihydrorhodamine 123 probe (DHR123, Sigma-Aldrich). Twenty-  
275 four hours or 21 days after seeding in black 96-well plates, cells were washed with PBS, then exposed  
276 for 45 min at 37°C to 1  $\mu$ M DHR123 prepared in FBS-free medium. Then, the DHR123 solution was  
277 replaced with PCL particles and incubated for 24h. Fluorescence was monitored at  $\lambda_{exc.}/\lambda_{em.}$   
278 480nm/530 nm, starting immediately after adding the exposure medium to the wells, then after 30  
279 min, 1h, 2h, 4h, 6h and 24h, using the Spectramax ID3 spectrofluorometer. Data are expressed as % of  
280 the fluorescence value of control (unexposed) cells. Tert-butyl hydroperoxide (TBHP, Sigma-Aldrich, 1  
281 mM) was used as positive control.

282

## 283 **2.9. DNA damage**

284 DNA strand breaks were quantified using the comet assay in alkaline condition, without  
285 formamidopyrimidine-DNA glycosylase (FPG) treatment. Cells were seeded in 12-well plates and  
286 exposed to PCL particles after 24 h or 21 days of differentiation. Methyl methanesulfonate (MMS, 30  
287  $\mu$ g/mL) was used as positive control. At the end of the treatment, cells were washed with PBS and  
288 harvested using trypsin-EDTA. Cells were washed with PBS and stored at -80°C in a cryoprotective  
289 solution (85.5 g/L sucrose, 11.76 g/L sodium citrate and 50 mL/L DMSO, pH 7.6), as such storage has  
290 been shown to preserve DNA integrity [50]. Glass slides were coated with 1% agarose and allowed to  
291 dry. Then, exposed cells were mixed with 1% low-melting agarose and deposited on the slides as two  
292 70  $\mu$ L gels per slide. After gel solidification, cells were lysed for 1 h in 2.5 M NaCl, 10 mM Tris, 0.1 M  
293 EDTA and 1% Triton X-100, pH 10 to release the DNA, then washed 3 times with PBS. Slides were  
294 immersed in cold electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH > 13) and incubated for 40 min,  
295 allowing the DNA to unwind. Then, a 1.2 V/cm current was applied to the slides for 30 min, to allow  
296 DNA migration. The slides were washed twice for 10 minutes in PBS and labelled for 20 min with 25  $\mu$ L  
297 GelRed™ (Biotium) diluted 1,000-fold in PBS. Comets were imaged on an Axioskop fluorescence  
298 microscope (Zeiss) and analyzed using Comet Assay IV (Perceptive Instruments). Fifty comets per gel

299 were analyzed, randomly chosen. The median value of % tail DNA was measured out of these 50  
300 comets. Each condition yielded six values (three technical replicates, with two gels for each of them),  
301 which were averaged.

302

#### 303 **2.10.** Intestinal barrier integrity (TEER and fluorescein passage)

304 For TEER measurements, cells were grown on polyester Transwell-clear inserts with a 0.4  $\mu\text{m}$  pore size  
305 (Costar, #3460) and maintained for 21 days post-confluence. Their culture medium was changed three  
306 times per week. TEER was monitored over these 21 days using an EVOM3 epithelial volt/ohm meter  
307 equipped with an ENDOHM-12 electrode (World Precision Instruments). After 21 days, cells were  
308 exposed to PCL particles for 24 hours, then the TEER of each insert was measured. EDTA  
309 (ethylenediaminetetraacetic acid, 2 mM) was used as positive control. The TEER from a blank insert  
310 (without cells) was subtracted to the measured values. The obtained values are expressed as % of the  
311 TEER of control (unexposed) cells. Following this, the exposure medium was replaced with medium  
312 containing 5  $\mu\text{g}/\text{mL}$  fluorescein (Sigma-Aldrich). After 2 h of incubation, 100  $\mu\text{L}$  of apical medium and  
313 100  $\mu\text{L}$  of basolateral medium were transferred to a black 96-well plate and fluorescence was  
314 measured on a Spectramax ID3 spectrofluorometer (Molecular Devices) at  $\lambda_{\text{exc.}}/\lambda_{\text{em.}}$  460 nm/515  
315 nm. Results were expressed as % fluorescence of control (unexposed) cells.

316

#### 317 **2.11.** Inflammation

318 Secretion of IL-8 was assessed by ELISA (Invitrogen, #88-7066-88). Cells were seeded in 12-well plates,  
319 grown for 24 h, then exposed for 24 h to PCL particles. At the end of exposure, exposure medium was  
320 harvested and stored at  $-80^{\circ}\text{C}$ . A transparent 96-well plate was incubated overnight at  $4^{\circ}\text{C}$  with the  
321 capture antibody, diluted in 0.1 M sodium carbonate. Then, the wells were washed three times with  
322 wash buffer (PBS 1X, 0.05% Tween<sup>®</sup>20) and non-specific binding sites were blocked for 1 h with 10%  
323 decompemented FBS prepared in PBS. A standard range was prepared using the IL-8 powder from the  
324 kit. After washing the plate three times, the standards and supernatants were deposited in the wells  
325 and incubated for 2 h at RT. After five washes, the detection antibody was added and incubated for 1  
326 h at RT. After five washes, the detection enzyme (peroxidase) was added and incubated for 30 min,  
327 followed by seven washes. Finally, the TMB substrate (3,3', 5,5' tetramethylbenzidine) was incubated  
328 for 30 min at RT in the dark. The reaction was stopped by adding 1 M sulphuric acid ( $\text{H}_2\text{SO}_4$ ).  
329 Absorbance was measured at 450 nm using a Spectramax ID3 (Molecular Device), with a background

330 correction at 570 nm. Absorbances values were converted to concentration using the IL-8 calibration  
331 curve.

332

### 333 **2.12.** Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

334 The impact of PCL particles on mRNA expression was assessed by RT-qPCR, targeting genes involved in  
335 oxidative stress, inflammation, endoplasmic reticulum stress, mucin secretion and xenobiotic efflux.  
336 Cells were seeded in 12-well plates and allowed to grow for 24 h. Then, they were exposed for 24 h to  
337 100 or 200 nm PCL particles. At the end of the treatment, cells were washed with PBS, lysed and their  
338 mRNA was extracted according to the supplier's protocol (NucleoSpin RNA, MACHEREY NAGEL,  
339 740955). It was reverse transcribed using SuperScript™ III (Invitrogen, 18080093). For qPCR, a reaction  
340 mix consisting of 20 ng of cDNA, 12.5 µL of Takyon No ROX SYBR MasterMix blue dTTP (Eurogentec),  
341 2.5 µL of 2 µM sense and antisense primers and nuclease-free water was prepared in a final volume of  
342 25 µL and deposited in the wells of a white 96-well qPCR plate. The plate was sealed with adhesive  
343 film, centrifuged for 5 min at 700 x *g*, and analysed using a CFX96 Touch Real-Time PCR Detection  
344 System (Biorad) with the following thermal cycle: 95°C for 5 min, 40 cycles of amplification 95°C for 15  
345 s, 55°C for 20 s, and 72°C for 40 s, then the melting curve: 95°C for 1 min, 55°C for 30 s, and 95°C for  
346 30 s. Results were analysed using REST© 2009 software [51]. The reference genes were  
347 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cyclophilin B (CycloB). Their sequences, as  
348 well as those of mRNA-specific primers, are reported in Table S1. Primer efficiency was tested prior to  
349 the experiment, being equal to  $2 \pm 0.3$ .

350

### 351 **2.13.** Statistical analysis

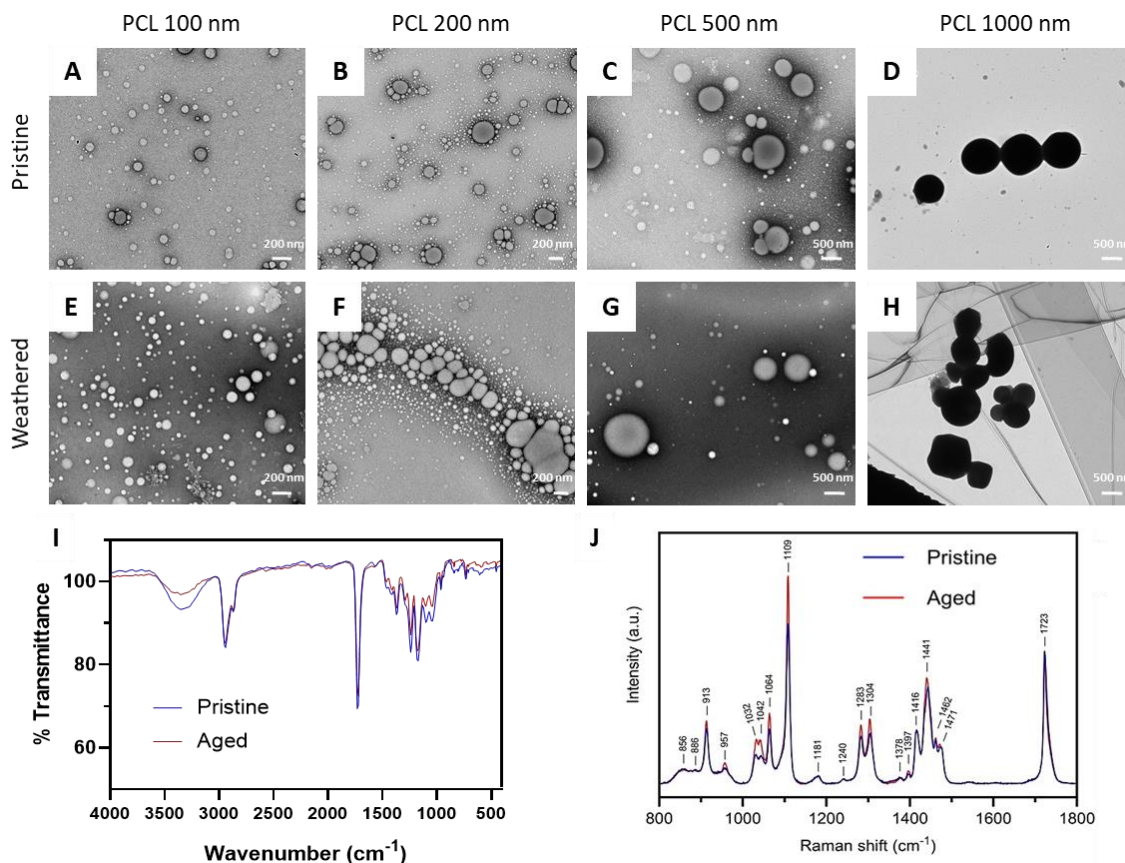
352 In all experiments except RT-qPCR, due to the small number of replicates in the experiments, data did  
353 not follow a normal distribution, variances were not homogeneous and the condition of  
354 homoscedasticity of residuals was not met. As a result, non-parametric statistical tests were carried  
355 out, i.e., Kruskal-Wallis tests, followed by two-tailed Dunn's post-hoc tests, using GraphPad Prism  
356 version 7. Results are considered statistically significant (\*) when the p-value is less than 0.05.

357

### 358 3. Results

#### 359 3.1. Artificial weathering and physicochemical characterization

360 According to the manufacturer's information, PCL particle diameters were 100, 200, 500 and 1000 nm.  
361 TEM analysis revealed that these particles were spherical but exhibited a rather heterogeneous size  
362 distribution. The measured primary diameters were  $78 \pm 22$  nm,  $204 \pm 87$  nm,  $505 \pm 182$  nm and  $939$   
363  $\pm 275$  nm, respectively (Figure 1A-D).



364 **Figure 1.** Physicochemical characterization of pristine and aged PCL particles. (A-H) TEM observations of pristine (A-D) and  
365 aged (E-H) PCL particles with diameter 100 nm (A, E), 200 nm (B, F), 500 nm (C, G) and 1000 nm (D, H). FTIR (I) and Raman (J)  
366 analysis of pristine (blue spectrum) and aged (red spectrum) 200 nm PCL particles.  
367

368 These observations were confirmed by DLS analysis, which showed mean Z-averages of  $105 \pm 5$  nm  
369 and  $197 \pm 2$  nm for the 100 and 200 nm particles, respectively, while their polydispersity index (PDI)  
370 was 0.17 (Table 1; size distributions are presented in Figure S1). This suggests size heterogeneity but  
371 mild agglomeration. Regarding 500 nm particles, the Z-average was  $363 \pm 60$  nm (PDI  $0.14 \pm 0.07$ ),  
372 which was significantly smaller than in the TEM measurement, likely due to the formation of  
373 aggregates exceeding  $1 \mu\text{m}$ . Such large particles are known to hamper proper measurement of smaller  
374 particles. The Z-average of 1000 nm particles could not be determined using this technique, which is

375 not well suited to measure particles with diameters > 1 $\mu$ m. PCL NPLs exhibited a slightly negative zeta  
 376 potential (Table 1). Following artificial weathering, morphological changes were observed, including  
 377 fusion of some of the particles which appeared less spherical (Figure 1E-F). However, particle fusion  
 378 was not frequent and did not lead to significant changes in primary diameter, Z-average or  
 379 polydispersity index, except for the 100 nm and 500 nm particles, for which the primary diameter was  
 380 15% larger (100 nm PCL NPLs) and 10% smaller (500 nm PCL NPLs) compared to the respective pristine  
 381 PCL NPLs (Table 1 and Figure S1). A slight decrease in zeta potential was observed; however, this trend  
 382 was not statistically significant (Table 1).

383

384 Table 1. Physico-chemical characteristics of pristine and weathered PCL particles<sup>a</sup>

	PCL 100 nm	PCL 200 nm	PCL 500 nm	PCL 1000 nm
<i>Primary diameter (nm)</i>				
Pristine (mean $\pm$ s.d.)	78 $\pm$ 22	204 $\pm$ 85	505 $\pm$ 182	939 $\pm$ 275
Pristine (50th [10th-90th] percentile)	73 [54-106]	190 [114-319]	473 [313-723]	881 [648-1239]
Aged (mean $\pm$ s.d.)	92 $\pm$ 43*	192 $\pm$ 79	458 $\pm$ 314*	937 $\pm$ 267
Aged (50th [10th-90th] percentile)	83 [52-148]	176 [120-276]	321 [168-911]	939 [571-1234]
<i>Z-average (nm)</i>				
Pristine	105 $\pm$ 5	197 $\pm$ 2	363 $\pm$ 60	<i>n.a.</i>
Aged	102 $\pm$ 2	186 $\pm$ 4	387 $\pm$ 13	<i>n.a.</i>
<i>Polydispersity index</i>				
Pristine	0.17 $\pm$ 0.03	0.17 $\pm$ 0.05	0.14 $\pm$ 0.07	<i>n.a.</i>
Aged	0.12 $\pm$ 0.02	0.17 $\pm$ 0.04	0.24 $\pm$ 0.01	<i>n.a.</i>
<i>Zeta (mV)</i>				
Pristine	-3.3 $\pm$ 3.9	-10.4 $\pm$ 1.1	-7.8 $\pm$ 4.4	-7.1 $\pm$ 4.5
Aged	-8 $\pm$ 2.5	-14.8 $\pm$ 1.9	-18.5 $\pm$ 0.3	-14.8 $\pm$ 0.8

385 <sup>a</sup> Primary diameter was determined from TEM observations (n=100), Z-averages and polydispersity indexes (Pdl) were  
 386 obtained from DLS analysis (n=3), zeta potential (Zeta) from ELS analysis (n=3). Statistical significance: \*p<0.05, aged vs.  
 387 pristine.

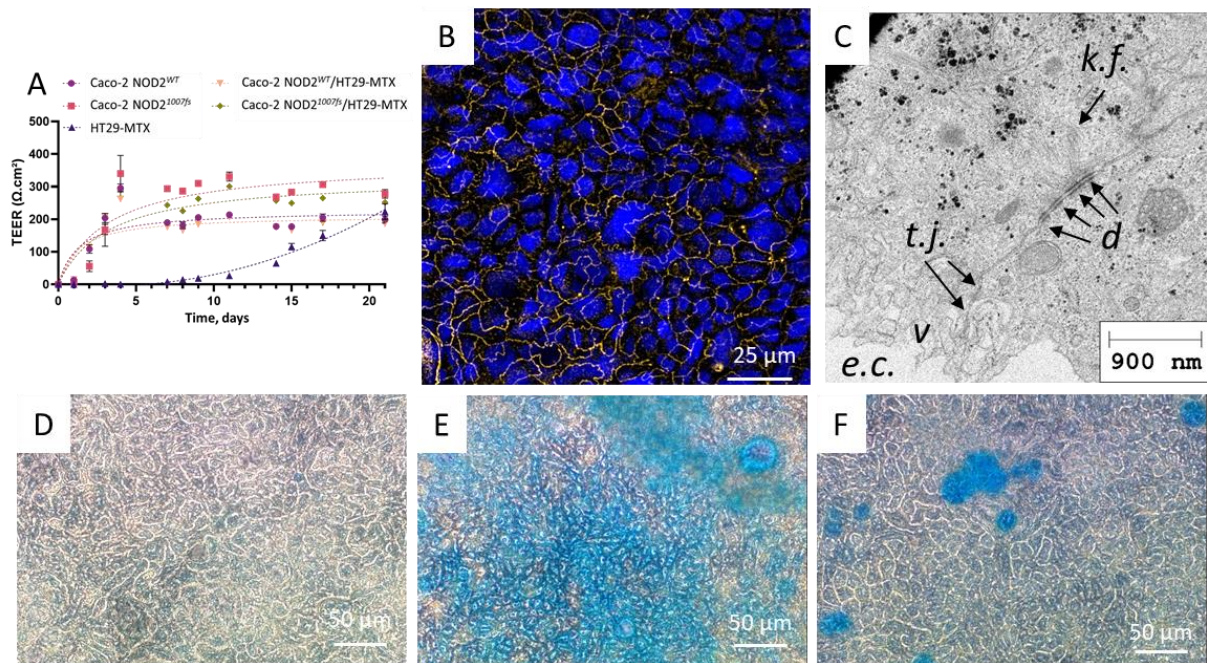
388

389 The chemical composition of PCL NPLs was confirmed via FTIR and Raman analysis. Indeed, the FTIR  
 390 spectrum recorded on 200 nm PCL particles revealed the characteristic features of PCL (Figure 1I).  
 391 These features included bands at 2944 cm<sup>-1</sup> and 2867 cm<sup>-1</sup>, corresponding to the asymmetric and  
 392 symmetric stretching of CH<sub>2</sub> groups, as well as ester-specific bands, particularly at 1723 cm<sup>-1</sup>, attributed  
 393 to the ester carbonyl (C=O) stretching, and at 1240 cm<sup>-1</sup> and 1186 cm<sup>-1</sup>, corresponding to asymmetric  
 394 and symmetric stretching of the C-O-C group. Weathering did not significantly change these FTIR  
 395 spectrums, as no new peak emerged and no characteristic peak disappeared. FTIR spectrums for 100  
 396 nm, 500 nm and 1000 nm PCL particles, reported in Figure S2, led to similar conclusions. Raman  
 397 analysis of 200 nm PCL NPLs revealed some changes in characteristic peaks after weathering,  
 398 compared to pristine NPLs. These changes included variations in the intensity of crystalline peaks at  
 399 913, 1032-1109, 1283-1304, and 1416-1471 cm<sup>-1</sup>, corresponding to the C-COO stretching, C-C  
 400 backbone stretching, CH<sub>2</sub> twisting, and CH<sub>2</sub> bending, respectively (Figure 1J).

401

### 402 3.2. Characterization of cell models

403 The phenotypic characterization of Caco-2 NOD2<sup>WT</sup> and Caco-2 NOD2<sup>1007fs</sup> has been described  
404 elsewhere [45]. In this study, the used cell models were co-cultures of these cells with HT29-MTX  
405 mucus-secreting cells. Such co-cultures were used either non-differentiated, i.e., exposed 24h after  
406 seeding, which is representative of a proliferative epithelium, or differentiated via growing them 21  
407 days post-confluence, which triggers their differentiation into a mature intestinal epithelium with tight  
408 junctions and microvilli [41]. Traditionally, Caco-2/HT29-MTX cell differentiation is performed on cells  
409 growing on semi-permeable inserts such as Transwell inserts. In the present study, they were grown  
410 on plastic or glass, therefore their differentiation status on these surfaces was examined.



411

412 **Figure 2.** Characterization of differentiated Caco-2 NOD2<sup>WT</sup> or 1007fs/HT29-MTX. Assessment of epithelium differentiation via  
413 measurement of the transepithelial electrical resistance (TEER) throughout the differentiation period (A). ZO-1  
414 immunostaining of Caco-2 NOD2<sup>WT</sup>/HT29-MTX after 21 days of differentiation (B). TEM image of Caco-2 NOD2<sup>WT</sup>/HT29-MTX  
415 exposed to 200 nm PCL NPLs (C), showing the presence of tight junctions (t.j.) and desmosomes (d) with associated keratin  
416 fibres (k.f.), as well as microvilli (v), which are hallmarks of epithelial cell differentiation. Mucus secretion visualized by Alcian  
417 blue staining in Caco-2 NOD2<sup>WT</sup> (D), HT29-MTX (E) and Caco-2 NOD2<sup>WT</sup>/HT29-MTX (F).

418 Differentiation into a tight epithelium was proven through TEER measurements and ZO-1  
419 immunostaining. After 21 days of culture post-confluence, Caco-2 NOD2<sup>WT</sup> exhibited a TEER of 199 ±  
420 11 Ω.cm<sup>2</sup>, whereas the Caco-2 NOD2<sup>WT</sup>/HT29-MTX co-culture showed a slightly lower resistance,  
421 reaching 187 ± 5 Ω.cm<sup>2</sup> (Figure 2A). Similarly, the TEER of Caco-2 NOD2<sup>1007fs</sup> was 278 ± 14 Ω.cm<sup>2</sup>, while  
422 that of the Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX co-culture was 252 ± 3 Ω.cm<sup>2</sup> (Figure 2A). Lower TEER of co-

423 cultures, as compared to monocultures, may be attributed to the presence of HT29-MTX cells, which  
424 show a much lower TEER compared to Caco-2 cells (Figure 2A).

425 ZO-1 immunostaining showed a uniform and continuous distribution along cell edges, indicating the  
426 presence of tight junctions (Figure 2B). Cells showed tight junctions, desmosomes and associated  
427 keratin fibres, as well as microvilli as observed by TEM (Figure 2C). Overall, this confirms that Caco-2  
428 NOD2<sup>WT</sup>/HT29-MTX differentiate into a tight epithelium, even when grown on plastic dishes rather  
429 than on transwell inserts. Mucus secretion was monitored by Alcian blue staining (Figure 2D-F). As  
430 expected, Caco-2 NOD2<sup>WT</sup> monoculture (Figure 2D) was negative for Alcian blue staining, indicating the  
431 absence of mucus secretion. In contrast, HT29-MTX monoculture (Figure 2E) and Caco-2  
432 NOD2<sup>WT</sup>/HT29-MTX co-culture (Figure 2F), produced a significant amount of mucus. Mucus covered  
433 HT29-MTX cell monoculture, but was not homogeneously distributed on top of the co-culture, likely  
434 locating only above HT29-MTX cells. The same was observed with Caco-2 NOD2<sup>1007fs</sup> cells (Figure S3A-  
435 B). Immunostaining showed cytoplasmic distribution of MUC5AC, specifically expressed by HT29-MTX  
436 cells (Figure S3C).

### 437 3.2. Internalization and accumulation of PCL particles and oligomers

438 Next, cells were exposed to PCL particles for accumulation and toxicity assessments. Throughout this  
439 study, we expressed the exposure concentration on a mass basis ( $\mu\text{g}/\text{mL}$  of exposure medium). Table  
440 S2 shows the corresponding concentration as number of particles per exposed well or as number of  
441 particles per cell, as calculated based on their size distribution.

442 Cellular accumulation of PCL particles was first assessed using flow cytometry, using 200 nm DB14-PCL  
443 fluorescently-labelled particles. After gating cells appropriately (Figure S4A), Control cells showed very  
444 low fluorescence (Figure S4B), while fluorescence of both Caco-2 NOD2<sup>WT</sup>/HT29-MTX (Figure S4C, S4E,  
445 S4G) and Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX (Figure S4D, S4F, S4H) increased with respect to exposure  
446 concentration, reflecting increased DB14-PCL intracellular accumulation. The mean fluorescence  
447 intensity (MFI) shifted from  $1.8 \pm 0.7$  fluorescence units (fu) in control (unexposed) cells to  $33.5 \pm 1.6$   
448 fu,  $56.4 \pm 0.3$  fu,  $65.5 \pm 0.6$  fu in Caco-2 NOD2<sup>WT</sup>/HT29-MTX cells exposed to 25, 50 or 100  $\mu\text{g}/\text{mL}$  DB14-  
449 PCL, respectively (Table 2). MFI in Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX cells increased from  $1.4 \pm 0.3$  fu in  
450 control cells to  $35.9 \pm 0.8$  fu,  $53.8 \pm 2.1$  fu and  $59.9 \pm 1.4$  fu in cells exposed to 25, 50 or 100  $\mu\text{g}/\text{mL}$   
451 DB14-PCL, respectively (Table 2), which did not significantly differ from the respective fluorescence of  
452 Caco-2 NOD2<sup>WT</sup>/HT29-MTX.

453

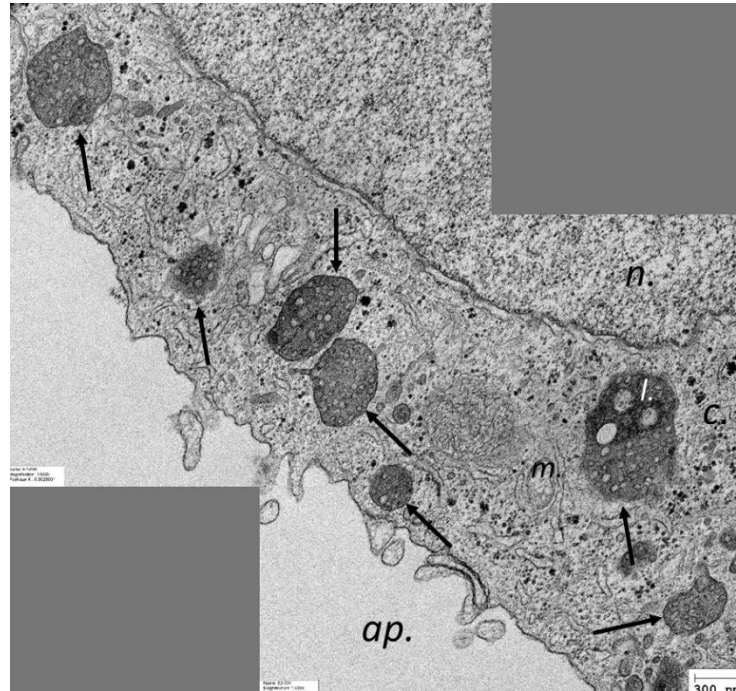
454

455 Table 2. Flow cytometry analysis of PCL-DB14 accumulation in Caco-2 / HT29-MTX cells<sup>a</sup>

PCL-DB14 concentration	Caco-2 NOD2 <sup>WT</sup> / HT29-MTX	Caco-2 NOD2 <sup>1007fs</sup> / HT29-MTX
0	1.8 ± 0.7	1.4 ± 0.3
25 µg/mL	33.5 ± 1.6*	35.9 ± 0.8*
50 µg/mL	56.4 ± 0.3* <sup>#</sup>	53.8 ± 2.1* <sup>#</sup>
100 µg/mL	65.5 ± 0.6* <sup>#</sup>	59.9 ± 1.4* <sup>#</sup>

456 <sup>a</sup> Mean fluorescence intensity (MFI) of cells exposed for 24 h to 25, 50 or 100 µg/mL PCL-DB14. Statistical significance:  
 457 \*p<0.05, exposed vs. control; <sup>#</sup>p<0.05, 50 µg/mL vs. 25 µg/mL or 100 µg/mL vs. 50 µg/mL; <sup>§</sup>p<0.05 Caco-2 NOD2<sup>WT</sup> / HT29-  
 458 MTX vs. Caco-2 NOD2<sup>1007fs</sup> / HT29-MTX: no statistical significance within these comparisons.

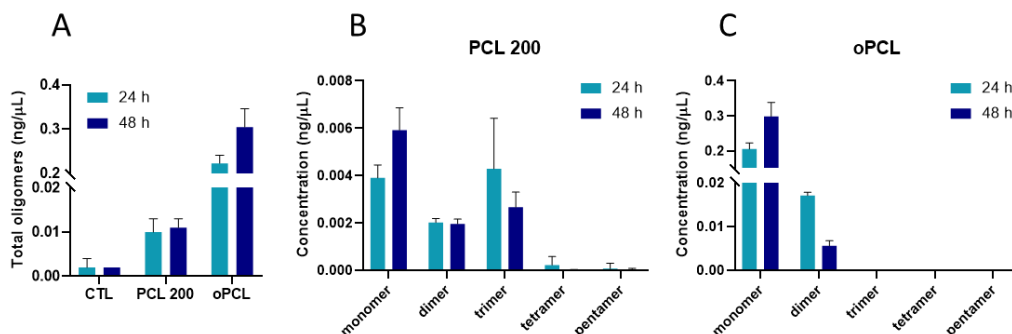
459 Then, PCL particle intracellular accumulation was probed by TEM imaging, confirming that PCL particles  
 460 accumulated in cells and located only in the cytoplasm, where they distributed in cytoplasmic vesicles  
 461 throughout the cells (Figure 3). PCL NPLs appeared light grey in these vesicles, because polymers are  
 462 not stained by osmium tetroxide and uranyl acetate. They showed spherical morphology and size  
 463 heterogeneity, which reflect the heterogenous size distribution of the used PCL particles as already  
 464 commented in section 3.1. No particle was observed in the cell nucleus or mitochondria in any of the  
 465 observed regions of the samples. The type of vesicles in which particles accumulated could not be  
 466 determined unambiguously, still they were always surrounded by a membrane and their relatively high  
 467 electron-density suggests that they were probably lysosomes.



468  
 469 **Figure 3.** TEM image of a Caco-2 NOD2<sup>WT</sup>/HT29-MTX cell exposed to 200 nm PCL particles. Two images are merged as a single  
 470 image, explaining why some grey regions are visible top right and down left corners of the image. Cells were exposed to 50  
 471 µg/mL of 200 nm pristine PCL NPLs for 24 h. This image shows the apical pole of the cell (ap.), with PCL particles accumulated

472 in electron-dense vesicles resembling lysosomes (arrows, *l.*) in the cell cytoplasm (*c.*). No PCL particle was observed in any of  
473 the observed cell nucleus (*n.*) or mitochondria (*m.*).

474 Anticipating the intracellular hydrolysis of internalized PCL particles, especially as they were  
475 accumulated in lysosomes where the pH is low and that contain a variety of enzymes aiming at  
476 degrading xenobiotics, we developed a HPLC-MS/MS method to quantify PCL oligomers in cell lysates,  
477 based on a highly sensitive multiple reaction monitoring (MRM) detection. Figure 4 illustrates PCL  
478 oligomer quantification in extracts from Caco-2 NOD2<sup>WT</sup>/HT29-MTX cells exposed for 24 or 48 hours  
479 to 200 nm PCL particles. As a comparison, cells were also exposed to a mixture of synthetic PCL  
480 oligomers (oPCL). The amount of oPCL to which cells were exposed corresponded to the amount of  
481 oligomers that would be released by the particles if they totally dissolved, as calculated according to  
482 their previously observed acellular dissolution behaviour [33]. Cells heavily accumulated oPCL, and  
483 intracellular accumulation increased as exposure time increased (Figure 4A). Intracellular oligomers  
484 could also be detected in cells exposed to PCL particles, although in quantities more than one order of  
485 magnitude lower than in cells exposed to oPCL. Their intracellular level remained stable over time  
486 (Figure 4A). With respect to oligomer length, the proportion of monomer, dimer, trimer and other  
487 polymer lengths differed in cells exposed to PCL particles, compared to cells exposed to oPCL. In cells  
488 exposed to PCL particles, after 24 h of exposure the same amount of monomer and trimer could be  
489 observed, as well as approximately half this amount of dimer. Some tetramers and pentamers could  
490 also be detected, although in minute quantities (Figure 4B). After 48h of exposure, the level of  
491 tetramers and pentamers decreased while the concentration of monomer increased, reaching 1.5-fold  
492 its amount at 24 h (Figure 4B). In cells exposed to oPCL, only monomers and dimers were detected at  
493 24 h, the monomer being predominant (Figure 4C). At 48h post-exposure, the amount of dimer  
494 decreased and the amount of monomer increased, suggesting that the dimer degraded inside the cells  
495 to form some monomers (Figure 4C).

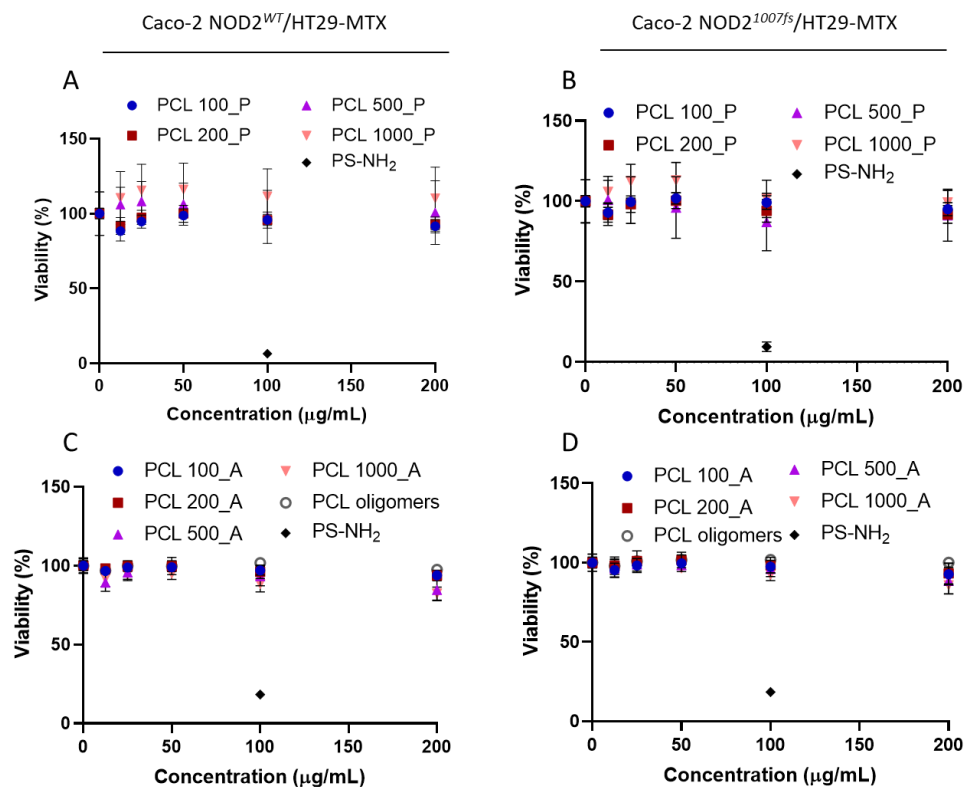


496

497 **Figure 4.** Quantification of PCL oligomers in Caco-2 NOD2<sup>WT</sup>/HT29-MTX extracts. Cells were exposed for 24 h or 48 h to 200  
498 nm pristine PCL NPLs (PCL200) (A, B) or to synthetic PCL oligomers (oPCL) (A, C). Cells were washed, then lysed, and PCL  
499 oligomers were quantified by HPLC-MS/MS in the filtered lysate. Mean ± standard deviation.

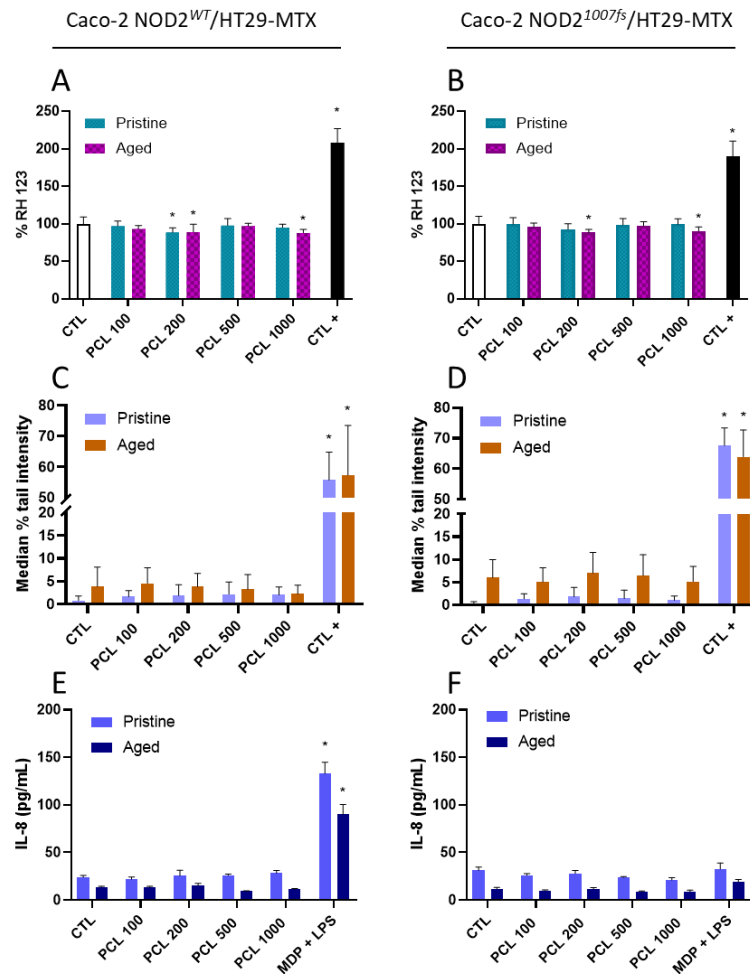
500 **3.3. Toxicity towards non differentiated Caco-2 NOD2<sup>WT</sup>/HT29-MTX or NOD2<sup>1007fs</sup>/HT29-MTX**

501 First, the toxicity of PCL particles was tested on Caco-2 NOD2<sup>WT</sup>/HT29-MTX and NOD2<sup>1007fs</sup>/HT29-MTX  
 502 24 h after their seeding, i.e., in a condition where they are still proliferative and not differentiated as  
 503 mature enterocytes. This mimics proliferative cells at the vicinity of intestinal crypts. When assessed  
 504 using the WST-1 assay, pristine (Figure 5A-B) and aged (Figure 5C-D) PCL particles did not alter the  
 505 viability of non-differentiated Caco-2 NOD2<sup>WT</sup>/HT29-MTX (Figure 5A, C) and Caco-2 NOD2<sup>1007fs</sup>/HT29-  
 506 MTX (Figure 5B, D), irrespective of their size and concentration. Similarly, PCL oligomers did not induce  
 507 cell death at the tested concentrations (Figure 5C-D). Moreover, neither pristine nor aged PCL particles,  
 508 regardless of their size, caused any increase in intracellular reactive oxygen species (ROS) levels in non-  
 509 differentiated Caco-2 NOD2<sup>WT</sup>/HT29-MTX (Figure 6A) or Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX (Figure 6B) after  
 510 24 h of exposure. A statistically significant decrease in fluorescence was observed, especially with 200  
 511 nm and 1000 nm PCL particles. This decrease was very weak, which questions its physiological  
 512 relevance. Figure S5 shows the monitoring of intracellular ROS from 30 minutes to 24 hours post-  
 513 exposure, showing no significant difference in exposed cells, compared to control cells, regardless of  
 514 the timepoint, cell model or particle type, i.e., pristine or aged.



515  
 516 **Figure 5.** Cytotoxicity of PCL particles and PCL oligomers to non-differentiated Caco-2 / HT29-MTX cells. Cytotoxicity was  
 517 assessed using the WST-1 assay in non-differentiated cells, i.e., 24 h after seeding, exposed for 24 h to pristine (A-B) or aged  
 518 (C-D) PCL particles or oligomers, on Caco-2 NOD2<sup>WT</sup>/HT29-MTX (A, C) and Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX (B, D). PS-NH<sub>2</sub> (50

519 nm, 100 µg/mL) were used as positive control. Results are expressed as % relative to control (unexposed cells). Mean ±  
 520 standard deviation.



521

522 **Figure 6.** Impact of PCL particles on intracellular ROS levels, DNA integrity and IL-8 secretion in non-differentiated Caco-2 /  
 523 HT29-MTX cells. Caco-2 NOD2<sup>WT</sup>/HT29-MTX (A, C, E) and Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX (B, D, F) were exposed for 24 h, 24 h  
 524 post-seeding, to 50 µg/mL pristine or aged PCL NPLs. Intracellular ROS levels were quantified using the DHR123 probe (A-B),  
 525 genotoxicity via the comet assay in alkaline conditions (no Fpg) (C-D) and inflammation by monitoring IL-8 secretion in the  
 526 exposure medium (E-F). Tert-butyl hydroperoxide (1 mM), methyl methanesulfonate (30 µg/mL) and a mixture of 10 µg/mL  
 527 MDP and 2 µg/mL LPS were used as positive controls (CTL+) in the DHR123, comet and IL-8 quantification assays, respectively.  
 528 Mean ± standard deviation; statistical significance: \*p<0.05, exposed vs CTL.

529 These results are consistent with the RT-qPCR analysis of gene expression, which showed no alteration  
 530 of the mRNA expression of genes involved in oxidative stress regulation, i.e., CAT, SOD1, SOD2, GCLM  
 531 and HO-1 (Table 3). The only observed changes were SOD-1 and GCLM mRNA expressions, which were  
 532 decreased in Caco-2 NOD2<sup>WT</sup>/HT29-MTX exposed to pristine 200nm PCL particles, as well as SOD-2 and  
 533 HO-1 mRNA expression that was slightly increased in Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX exposed to 100 nm  
 534 pristine PCL particles (Table 3). This suggests homeostatic regulation, which did not lead to overt  
 535 oxidative stress as no excess ROS were detected.

536

537 **Table 3.** RT-qPCR analysis of mRNA expression in non-differentiated Caco-2 NOD2<sup>WT</sup>/HT29-MTX and Caco-2  
 538 NOD2<sup>1007fs</sup>/HT29-MTX cells<sup>a</sup>

	WT				1007fs			
	100 nm pristine	100 nm aged	200 nm pristine	200 nm aged	100 nm pristine	100 nm aged	200 nm pristine	200 nm aged
<i>Ox. stress</i>								
CAT	-	-	-	-	-	-	-	-
SOD1	-	-	0.52±0.10	-	-	-	-	-
SOD2	-	-	-	-	-	-	-	-
GCLM	-	-	0.59±0.10	-	-	-	-	-
HO-1	-	-	-	-	1.76±0.51	-	-	-
<i>Inflammation</i>								
IL-1β	-	-	-	-	-	-	-	-
IL-8	-	-	1.93±0.38	-	-	-	-	-
TNFα	-	-	-	-	1.88±0.51	-	-	-
<i>ER stress</i>								
ATF6	1.56±0.24	1.69±0.27	1.30±0.19	2.02±0.51	-	-	-	-
GRP78	-	-	-	-	3.00±0.88	3.37±0.86	-	2.53±0.78
IRE1	-	-	-	-	1.85±0.49	-	-	-
sXBP1	-	-	-	-	1.83±0.55	-	-	-
CHOP	-	-	-	-	2.50±0.65	2.71±0.66	-	2.04±0.53
<i>Mucins</i>								
MUC2	-	-	0.51±0.10	0.32±0.07	-	-	-	-
MUC5AC	-	-	0.71±0.13	-	-	-	-	-
<i>Afflux pumps</i>								
MDR1	-	-	-	1.77±0.36	-	-	-	-
MRP1	1.39±0.17	-	-	-	-	-	-	-
MRP2	-	-	-	-	-	-	-	-
BCRP	-	-	-	-	-	-	-	-

539 <sup>a</sup>mRNA expression normalized using the  $\Delta\Delta C_t$  method, analysed in Caco-2 NOD2<sup>WT</sup>/HT29-MTX cells (WT) and in Caco-2  
 540 NOD2<sup>1007fs</sup>/HT29-MTX cells (1007fs), exposed to 50  $\mu\text{g}/\text{mL}$  of 100 nm or 200 nm PCL particles, pristine or aged. Only  
 541 statistically significant results are reported, non-significant data are represented by a dash. Absolute gene regulation  $\pm$   
 542 standard error, as calculated using REST2009 [51].

543

544 Similarly, no significant increase of DNA strand breaks and/or alkali-labile sites was observed in non-  
 545 differentiated cultures of Caco-2 NOD2<sup>WT</sup>/HT29-MTX (Figure 6C, 50  $\mu\text{g}/\text{mL}$  and Figure S6, all tested  
 546 concentrations) and Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX (Figure 6D, 50  $\mu\text{g}/\text{mL}$  and Figure S6, all tested  
 547 concentrations), exposed to pristine or aged PCL particles, whatever their diameter. Finally, no  
 548 significant increase in IL-8 secretion was observed after exposure of non-differentiated Caco-2  
 549 NOD2<sup>WT</sup>/HT29-MTX (Figure 6E, 50  $\mu\text{g}/\text{mL}$  and Figure S7, all tested concentrations) and Caco-2  
 550 NOD2<sup>1007fs</sup>/HT29-MTX (Figure 6F, 50  $\mu\text{g}/\text{mL}$  and Figure S7, all tested concentrations) to PCL particles,  
 551 pristine or aged, whatever their size. Conversely, co-exposure to MDP at 10  $\mu\text{g}/\text{mL}$  and LPS at 2  $\mu\text{g}/\text{mL}$   
 552 induced IL-8 secretion in Caco-2 NOD2<sup>WT</sup>/HT29-MTX but not in Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX cells. It  
 553 was expected since MDP is the natural ligand for NOD2, and NOD2 does not recognize MDP when  
 554 carrying the 1007fs mutation. At the mRNA expression level, signs of inflammation were observed only  
 555 in Caco-2 NOD2<sup>WT</sup>/HT29-MTX exposed to pristine 200 nm PCL particles, where IL-8 mRNA expression

556 was significantly increased (Table 3) and in Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX exposed to pristine 100 nm  
557 PCL, where increased mRNA expression of TNF- $\alpha$  was measured (Table 3).

558 Finally, the impact of PCL NPLs on the expression of mRNAs encoding proteins involved in the response  
559 to endoplasmic reticulum (ER) stress, i.e., the unfolded protein response (ATF6, GRP78, IRE1, sXBP1  
560 and CHOP), mucin secretion (MUC2 and MUC5AC) and xenobiotic efflux pumps belonging to the ABC  
561 transporter family (MDR1, MRP1, MRP2 and BCRP) was analyzed by RT-qPCR. All mRNA relative  
562 expression results are reported in Table 3. The most affected pathway was ER stress, with ATF-6 mRNA  
563 expression that was increased in all tested conditions in Caco-2 NOD2<sup>WT</sup>/HT29-MTX, whereas in Caco-  
564 2 NOD<sup>1007fs</sup>/HT29-MTX pristine 100 nm PCL particles significantly increased GRP78, IRE1, sXBP1 and  
565 CHOP mRNA expression. GRP78 and sXBP1 mRNA expression was also increased in Caco-2  
566 NOD<sup>1007fs</sup>/HT29-MTX exposed to aged 100 nm and 200 nm PCL particles. Moreover, mRNA expression  
567 of some xenobiotic efflux pumps was also significantly affected, with MRP1 expression that was  
568 increased in Caco-2 NOD2<sup>WT</sup>/HT29-MTX exposed to 100 nm or 200 nm pristine PCL particles, while  
569 MDR1 expression was also increased in Caco-2 NOD2<sup>WT</sup>/HT29-MTX exposed to 200 nm pristine PCL  
570 particles. No significant change was observed in Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX cells. In the mucus  
571 secretion pathway, no significant change was observed in Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX cells, while  
572 pristine 100 nm PCL increased the mRNA expression of MRP1 and aged 200 nm PCL particles increased  
573 the mRNA expression of MDR1 in Caco-2 NOD2<sup>WT</sup>/HT29-MTX.

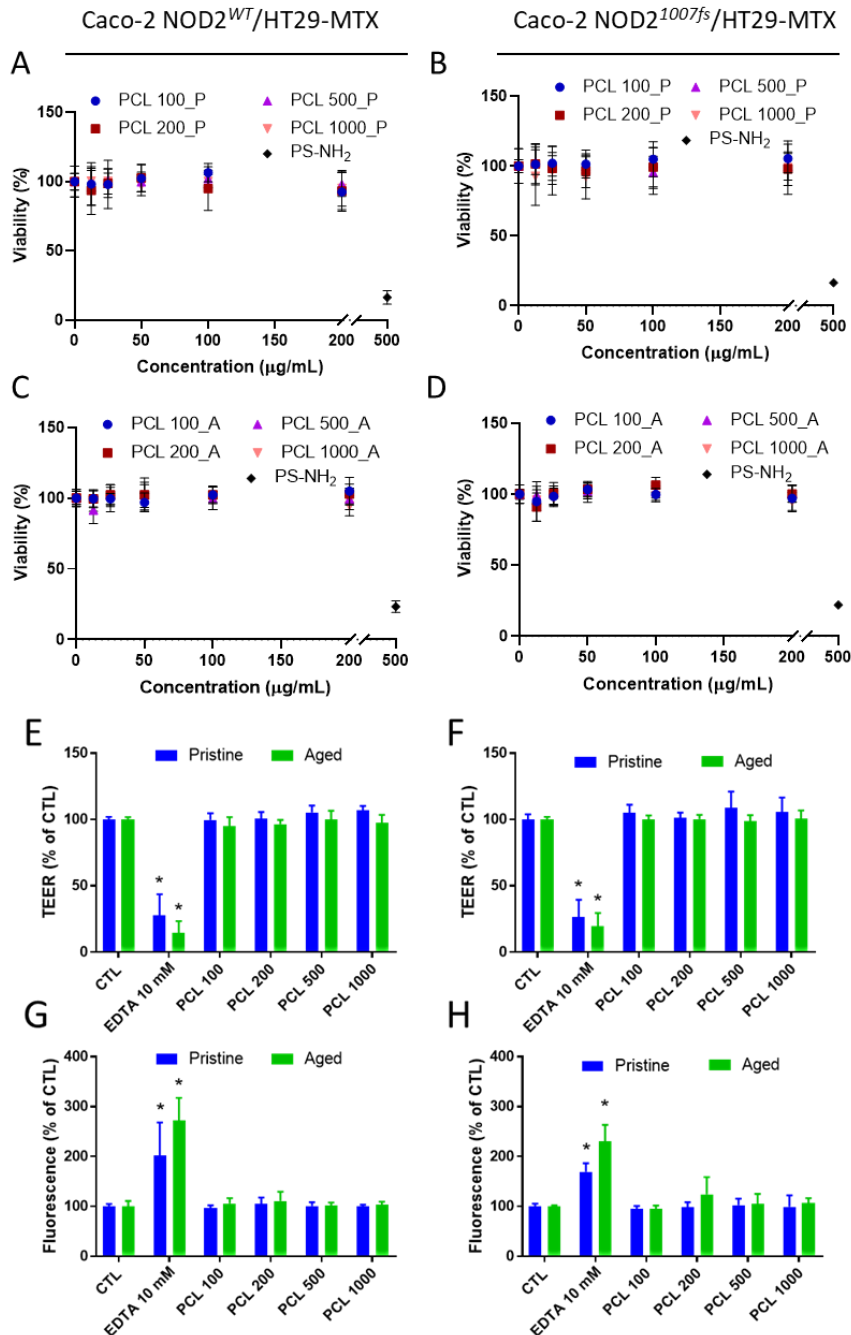
574

#### 575 **3.4. Toxicity towards differentiated Caco-2 NOD2<sup>WT</sup> or NOD2<sup>1007fs</sup>/HT29-MTX**

576 Then, the toxicity of PCL particles was assessed on differentiated Caco-2/HT29-MTX cells, in which  
577 Caco-2 cells are differentiated into mature enterocytes with tight junctions and villi, therefore being  
578 more representative of human intestinal epithelium. Differentiated Caco-2/HT29-MTX cells are known  
579 to be more resistant to particles than undifferentiated ones, owing to the presence of microvilli that  
580 would hamper particle uptake by endocytosis. Pristine and aged PCL, whatever their size, did not affect  
581 the viability of differentiated Caco-2 NOD2<sup>WT</sup>/HT29-MTX and Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX cells, which  
582 remained close to 100% whatever the particle concentrations and size (Figure 7A-D). Then, the impact  
583 of these particles on the intestinal barrier integrity was assessed by measuring the TEER and the  
584 transepithelial passage of fluorescein, which is a high molecular weight fluorescent dye that crosses  
585 the epithelial barrier via the paracellular spaces only if tight junctions are compromised. PCL particles  
586 did not alter the integrity of the epithelial barrier, with TEER remaining unchanged, whereas EDTA,  
587 used as a positive control, significantly reduced it (Figure 7E-F). It was confirmed by the absence of

588 fluorescein passage after exposure to pristine or aged PCL particles, unlike the positive control, EDTA  
 589 (Figure 7G-H).

590

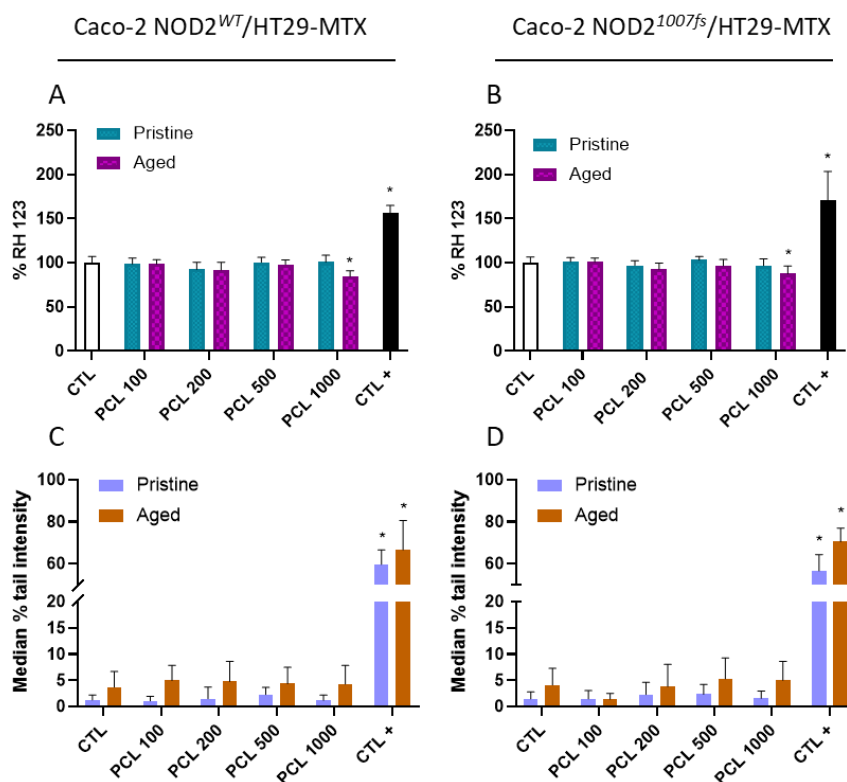


591

592 **Figure 7.** Impact of PCL particles on cell viability and intestinal barrier integrity, in differentiated Caco-2 / HT29-MTX cells.  
 593 Cells were grown post-confluence for 21 days, then exposed for 24 h to PCL NPLs. Cytotoxicity, as assessed using the WST-1  
 594 assay (A-D), after exposure to pristine (PCL\_P, A, B) or aged (PCL\_A, C, D) PCL NPLs, of Caco-2 NOD2<sup>WT</sup>/HT29-MTX (A, C) and  
 595 Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX (B, D). TEER measurement (E-F) and fluorescein paracellular passage (G-H) through Caco-2  
 596 NOD2<sup>WT</sup>/HT29-MTX (E, G) and Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX (F, H), exposed to 50 μg/mL pristine (blue) and aged (green) PCL  
 597 NPLs. PS-NH<sub>2</sub> 50 nm particles (500 μg/mL) and EDTA (10 mM) were used as positive controls for the WST-1 and

598 TEER/fluorescein permeability assays, respectively. Mean  $\pm$  standard deviation; statistical significance: \* $p < 0.05$ , exposed vs  
 599 CTL.

600 None of the PCL particles investigated, whether pristine or aged, and regardless of their size, induced  
 601 any increase in intracellular ROS levels after 24 h of exposure compared to the control, neither in Caco-  
 602 2 NOD2<sup>WT</sup>/HT29-MTX (Figure 8A) for the 24 h time-point and Figure S8 for all other tested time-points)  
 603 nor in Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX cells (Figure 8B for the 24 h time-point and Figure S8 for all other  
 604 tested time-points), except aged 1000 nm PCL that caused a small but significant decrease of ROS  
 605 levels. This decrease was small (~10%), which questions its physiological relevance. Neither did PCL  
 606 particles, either pristine or aged, induce any DNA strand breaks as assessed via the comet assay in  
 607 alkaline conditions, whatever their size and the cell model (Figure 8C-D and Figure S9).



608  
 609 **Figure 8.** Impact of PCL particles on intracellular ROS levels and DNA integrity in differentiated Caco-2 / HT29-MTX cells. Cells  
 610 were grown for 21 days post-confluence, then exposed for 24 h to 50  $\mu$ g/mL PCL NPLs. Intracellular ROS levels (A-B) and DNA  
 611 strand breaks (C-D) triggered by pristine and aged PCL NPLs with primary diameters 100 nm (PCL 100), 200 nm (PCL 200), 500  
 612 nm (PCL 500) or 1000 nm (PCL 1000). Tert-butyl hydroperoxide (1 mM) and methyl methanesulfonate (30  $\mu$ g/mL) were used  
 613 as positive controls for the DHR123 and comet assay, respectively. Mean  $\pm$  standard deviation; statistical significance:  
 614 \* $p < 0.05$ , exposed vs CTL.

615  
 616

#### 617 4. Discussion

618

619 Biodegradable plastics, such as PCL, have been developed to improve waste management and they  
620 find applications in packaging, 3D printing, and in the medical sector due to their attractive properties  
621 [52]. Although PCL is FDA-approved for its biocompatibility, its slow degradation in the body raises  
622 concerns about the potential release of MNPs and oligomers that could affect health. Some studies  
623 highlighted that PCL are cytotoxic and/or dysregulate immune cell inflammatory response [28-30].  
624 Unlike most studies evaluating the impact of only pristine particles, which are not representative of  
625 real-world conditions, this study assessed PCL particles in their pristine state, as well as after artificial  
626 aging in a UV test chamber, mimicking sunlight and temperature on a sunny day at noon in the  
627 equatorial region [32]. The physicochemical changes induced by particle weathering should be  
628 considered in toxicological studies since nanoparticle toxicity mechanisms are closely linked to their  
629 physical and chemical properties [31]. We selected particles with sizes ranging from 100 to 1000 nm,  
630 as the toxicity of particles made from other polymers, such as polystyrene, is known to be size-  
631 dependent [21, 53, 54].

632 As expected, weathering only slightly affected the shape, size, and surface charge of PCL particles, with  
633 significant primary diameter changes restricted to the 100 nm (increase) and 500 nm (decrease)  
634 fractions, and a slight shift toward more negative zeta potentials that did not reach statistical  
635 significance. However, we previously reported that the same weathering conditions promoted the  
636 release of soluble PCL oligomers of varying sizes, from the monomer to the pentamer, thus confirming  
637 the sensitivity of this polyester to hydrolysis [33, 34]. Moreover, Raman spectroscopy revealed an  
638 increase in the crystallinity of the 200 nm particles after aging, with an intensification of crystalline  
639 peaks [55, 56]. This variation could result either from polymer annealing, favouring chain  
640 reorganization, or from the degradation of amorphous regions by hydrolysis, leaving only the  
641 crystalline areas. The latter hypothesis is supported by several studies that have observed increased  
642 PCL crystallinity after chemical or enzymatic hydrolysis [57-60].

643 PCL toxicity was assessed on *in vitro* cell models based on Caco-2 cells, which is commonly used in  
644 intestinal toxicology [61, 62]. These cells were genetically engineered by introduction of NOD2 variants  
645 to be representative of healthy individuals (Caco-2 NOD2<sup>WT</sup>) and of more sensitive individuals, i.e.,  
646 people showing genetic susceptibility to develop CD (Caco-2 NOD2<sup>1007fs</sup>), for which some  
647 environmental risk factors have been described and among them exposure to particulate matter [63].  
648 We hypothesized that plastic particles could contribute to the development or exacerbation of this  
649 disease. This hypothesis is supported by the work of Yan et al., who found a positive correlation  
650 between the concentration of MNPs in faeces and the severity of IBD [15], as well as by other research

651 showing an exacerbation of symptoms in mice with colitis after exposure to MNPs [24, 25]. To enhance  
652 the biological relevance of the model, Caco-2 cells were co-cultured with HT29-MTX cells, which  
653 secrete some mucus. The study explored the effect of particles on co-cultures at different stages of  
654 differentiation, thereby modelling their impact on non-differentiated intestinal epithelium, similar to  
655 cells located in intestinal crypts, and differentiated epithelium, characterized by tight junctions and  
656 mucus production.

657 We demonstrated the accumulation of PCL particles in cells, probably by endocytosis in agreement  
658 with studies showing the internalization of PS particles with similar size and surface charge in Caco-2  
659 cells [61, 64]. These results are also consistent with other studies reporting the uptake of PCL MNPs by  
660 various cell types [28-30]. An interesting consequence of PCL particle internalization is the presence of  
661 oligomers in the intracellular compartment. These oligomers could result from particle degradation or  
662 from the internalization of oligomers that had formed during the exposure time in the exposure  
663 medium, as we previously demonstrated that PCL particles degraded in cell culture medium due to the  
664 presence of foetal bovine serum [33]. If oligomers detected in the cell extracts were arising from the  
665 internalization of hydrolysis products produced within the exposure medium, the yield of particle  
666 degradation in the exposure medium would have to be around 5 % at 24h, which is more than twice  
667 the yield that we previously observed, i.e., lower than 2% at 96h [33]. Moreover, the proportion of  
668 monomers observed in cells exposed to PCL particles was 24% and 35% after 24h and 48 h,  
669 respectively, while in cells exposed to oPCL it was 3 to 4 times higher, with 92% and 98% at 24 h and  
670 48 h, respectively. Therefore, this rules out a major contribution of internalization of oligomers  
671 produced within the culture medium and rather suggests that these oligomers originate from PCL  
672 particle degradation inside cells. An interesting feature of the detected hydrolysis products is the  
673 predominance of short-size oligomers, with the monomer being the major species. This contrasts with  
674 results obtained upon aging in water where the pentamer and tetramer were the most frequent  
675 products. These observations suggest that esterases and lipases produced by Caco-2 cells are actively  
676 involved in the intracellular hydrolysis of PCL [65-69].

677 It is essential to evaluate the toxicity of the released oligomers, which may differ from that of the intact  
678 particles, as has been observed with other plastics, including PS, PC and PVC, containing toxic  
679 monomers with detrimental effects on human health [70]. Although our results confirm the  
680 accumulation of PCL particles in cells and their degradation into oligomers, no toxicity was observed,  
681 whatever the particle size, in terms of cell viability, ROS production, epithelial barrier integrity, DNA  
682 integrity, or IL-8 secretion. Synthetic oligomers did not cause any cytotoxicity as well. These findings  
683 hold true regardless of cell differentiation or genetic susceptibility to CD. However, some alterations  
684 in mRNA expression were observed, proving that cells responded to PCL particle exposure, although

685 in a homeostatic manner. Indeed, exposure to PCL particles induced endoplasmic reticulum stress  
686 response, leading to the modulation of unfolded protein response markers, as previously  
687 demonstrated in mice exposed to PS particles [71]. Disruption of protein folding can compromise  
688 cellular homeostasis and, if adaptive mechanisms fail, it would trigger apoptosis [72]. In cells  
689 expressing wild-type NOD2, ATF6 was induced, while cells expressing NOD2<sup>1007fs</sup> exhibited  
690 overexpression of GRP78 and sXBP1, suggesting a link to CD. Indeed, elevated levels of GRP78 and  
691 sXBP1 have been detected in the inflamed intestines of patients with CD, suggesting that ER stress  
692 promotes chronic inflammation [73]. Moreover, significant changes in endoplasmic reticulum proteins  
693 levels have also been reported in macrophages exposed to 200 nm PCL particles [28]. We also observed  
694 that exposure to PCL particles altered the expression of ABC transporters, notably MDR1 (i.e., the P-  
695 gp) and MRP1, in line with studies showing that other particle internalization can impact these  
696 transporters [53, 74]. ABC transporters play key roles in the efflux of xenobiotics and in cellular  
697 protection [75]. Still, these alterations should be confirmed at the protein level (e.g., by Western blot  
698 or proteomics) or at the functional level, and ideally validated in more physiologically relevant models,  
699 such as primary cells or organoid models.

700 To our knowledge, no study has yet examined the effects of PCL NPLs on Caco-2/HT29-MTX intestinal  
701 cells. However, our observations are consistent with studies conducted on other NPLs, such as PS, PP,  
702 and PA, which report little to no toxic effects on these human intestinal cells, regardless of their level  
703 of differentiation [53, 61, 64, 76-78]. Similarly, recent studies conducted in our laboratory showed that  
704 PS-COOH, PET and PLA NPLs do not induce any toxic effects on the same intestinal models as those  
705 used in this study [44, 45]. However, it is important to note that the effects of NPLs vary considerably  
706 between *in vitro* and *in vivo* studies. Research has shown that various NPLs (PS, PE, PP, PA, and PVC)  
707 cause significant intestinal effects in the zebrafish, *C. elegans*, and mice [17-19, 79, 80]. For example,  
708 Jin et al. showed that exposure to PS NPLs caused dysbiosis, intestinal barrier dysfunction, and  
709 metabolic disorders in mice [20]. Similarly, Dussol et al. observed increased sensitivity to PS-COOH  
710 NPLs in *Nod2*<sup>KO</sup> mice compared to *Nod2*<sup>WT</sup> mice, while no impact was observed on Caco-2 (NOD2<sup>WT</sup> or  
711 NOD2<sup>1007fs</sup>) / HT29-MTX *in vitro* models [45]. These results suggest that individuals with genetic  
712 susceptibility to CD may be more affected by exposure to NPLs than healthy individuals. They highlight  
713 the lower sensitivity of Caco-2/HT29-MTX co-cultures compared to *in vivo* mouse models [45]. An  
714 important caveat of these epithelial models is the use of Caco-2 and HT29-MTX cancer-derived cell  
715 lines, which may not fully reproduce the phenotype, differentiation state, barrier physiology and stress  
716 responses of primary intestinal epithelium, especially when considering their genotype, which is not  
717 normal. The properties of mucus produced by HT29-MTX remain an approximation of physiological  
718 intestinal mucus [81, 82]. Moreover, it would be more suitable to evaluate ER stress on cells that would

719 produce and manufacture large amounts of proteins such as Goblet cells or Paneth cells, instead of  
720 epithelial cells [83]. Beyond these cell line-related considerations, such *in vitro* models lack  
721 representativeness of the intestine, particularly because they do not include any immune cells and  
722 microbiota. The latter is a drastic difference between *in vitro* and *in vivo* models. Indeed, the  
723 microbiota plays a crucial role in intestinal homeostasis and is influenced by various environmental  
724 factors, such as diet and medication [84]. Many *in vivo* studies have shown that exposure to NPLs  
725 causes intestinal disturbances, including dysbiosis [18, 85]. This imbalance can impair the intestinal  
726 barrier, promote chronic inflammation, and contribute to the development of diseases such as IBD  
727 [86]. A recent study showed that PS NPLs induce dysbiosis, oxidative stress, intestinal damage, and  
728 systemic inflammation in mice. These effects were significantly attenuated in microbiota-depleted  
729 mice, suggesting that the microbiota may mediate the toxicity of PS NPLs on the intestine [21]. These  
730 results align with the work of Qiao et al., who highlighted a major indirect impact of dysbiosis in the  
731 intestinal barrier dysfunction induced by PS NPLs in mice, thereby emphasizing the central role of the  
732 microbiota in the toxicity of NPLs [87]. Finally, the model does not include digestion simulation, where  
733 pH variations and other physicochemical conditions may alter particle properties and their interactions  
734 with the epithelium [88, 89].

735 Thus, to overcome these limitations, it would be relevant to integrate the microbiota as well as  
736 immune cells, which are essential for studying the symptoms of IBD, and to pre-condition particles by  
737 passing them through simulated digestive fluids to better reproduce gastrointestinal tract conditions.  
738 The development of more complex intestinal models, such as organoids or primary cells, would allow  
739 for the *in vitro* reproduction of an environment closer to the *in vivo* intestine [90]. The use of models  
740 combining human intestinal epithelial cells, immune cells, and the microbiota, for example on  
741 microfluidic chips, would be a promising approach to assess the effects of plastic NPLs on the intestine  
742 [91]. Beyond improving the physiological relevance of the model, refining the exposure design is also  
743 important to better capture response dynamics. Most endpoints were assessed after a single 24 h  
744 exposure, although intracellular accumulation was observed. For potentially accumulating materials,  
745 a 24 h exposure may be insufficient to rule out transient, delayed, or cumulative responses; therefore,  
746 the present data primarily support conclusions on acute effects. Future work would benefit from  
747 incorporating kinetic measurements (e.g., 0, 6, 12, 24, 48, and 72 h) and/or longer or repeated  
748 exposure regimens (e.g., daily re-dosing with media renewal over several days) to better capture  
749 accumulation-driven responses.

750 Another outcome of this work is that aging particles did not increase their toxicity on intestinal models,  
751 which is often reported in the literature. For example, a study on PS particles, either pristine or aged  
752 by UV irradiation, highlighted a higher cytotoxicity of the oxidized particles on Caco-2 cells [92].

753 Similarly, PCL particles aged by alkaline treatment (NaOH), inducing hydrolysis and generating reactive  
754 groups (carboxyl and hydroxyl), caused increased toxicity, in terms of cell viability and reduced  
755 hemocompatibility [29]. Furthermore, PCL oligomers did not show cytotoxicity, which is consistent  
756 with a study indicating low toxicity of 6-hydroxyhexanoic acid on fibroblasts [93], but contrasts with  
757 another study showing that PLA oligomers caused intestinal damage and acute inflammation in mice  
758 [94]. However, in this latter case, the final degradation product of PLA oligomers is lactic acid. Excess  
759 of this glycolysis end by-product may thus perturb metabolic networks more than longer hydroxy acids  
760 such as 6-hydroxyhexanoic acid. Comparing these studies is complex due to the diversity of aging  
761 protocols, the types of NPLs and oligomers analysed, the characterization methods, and the models  
762 and toxicity tests employed. This heterogeneity makes the assessment of NPLs' hazard challenging.  
763 Therefore, it is crucial to standardize NPLs toxicity studies to ensure reliable and scientifically sound  
764 conclusions, as recommended by several authors [32, 95].

765

## 766 **5. Conclusion**

767

768 This study assessed the toxic effects of PCL NPLs on human colon epithelium models, *in vitro*, taking  
769 into account their aging in conditions mimicking a journey in the environment. Our results show that  
770 aging modifies the physicochemical properties of the particles, leading to increased crystallinity and  
771 the release of oligomers through hydrolysis. Both pristine and aged PCL NPLs did not trigger any toxicity  
772 in the assessed exposure conditions, regardless of particle size, their pristine or aged state, or cell  
773 phenotype. PCL NPLs accumulated in these cells without affecting their viability, ROS production, DNA  
774 integrity, epithelial barrier integrity or IL-8 secretion. However, changes in the expression of some  
775 endoplasmic reticulum stress markers and ABC transporters were detected, suggesting subtle  
776 responses to PCL NPL exposure. These results align with studies on other NPLs, showing low toxicity,  
777 *in vitro*, upon acute exposure. However, the limitations of the models used, particularly the absence  
778 of microbiota and immune cells, and the focus on colon cells only, highlight the need for more complex  
779 systems to better understand the effects of NPLs on the intestine. Finally, the heterogeneity of  
780 experimental protocols emphasizes the importance of standardizing studies to better understand the  
781 impact of MNPs on human health.

782

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802

## 803 **8. Conflict of interest**

804 The authors declare no conflict of interest

805

## 806 **9. Authors contribution**

807 Investigation: MB, VB, MP, DB, SD, DF, ED, VCF, TR, TD; Formal analysis: MB, VB, MP, SD, VCF, TD.  
808 Conceptualization, Methodology, Validation, Supervision: MC, TD; Visualization: MB, VB, MP, DB, DF,  
809 CM, TD, MC; Funding acquisition, Resources, Project administration: MC; Writing-original draft: MB,  
810 MC, Writing-review and editing: MB, TR, TD, MC.

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## Supplementary Materials

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1054 **Table S2. RT-qPCR primers sequences**

	Forward primer (5'>3')	Reverse primer (5'>3')
<i>Reference genes</i>		
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
CYCLOB	GCAAGATCGAGGTGGAGAAG	CTGTGGAATGTGAGGGGAGT
<i>Oxidative stress</i>		
CAT	AGCTTAGCGTTCATCCGTGT	TCCAATCATCCGTCAAAACA
SOD1	AGGGCATCATCAATTTGAG	ACATTGCCCAAGTCTCCAAC
SOD2	TCCACTGCAAGGAACAACAG	TCTTGCTGGGATCATTAGGG
GCLM	AGTCCTTGAGTTGCACAGC	ACACAGCAGGACGCAAGATT
HO-1	TTCTCCGATGGGTCTTACACT	GGCATAAAGCCCTACAGCAACT
<i>Inflammation</i>		
IL-1 $\beta$	CTGCCAGTGAAATGATGGCT	TAGGTGCGATGCTTAGAGGCTG
IL-8	TGGCAGCCTTCTGATTTCT	ATTTCTGTGTTGGCGCAGTG
TNF $\alpha$	GGCGTGGAGCTGAGAGATAAC	GGTGTGGGTGAGGAGCACAT
<i>Endoplasmic reticulum stress</i>		
GRP78	GGTGAAAGACCCCTGACAAA	GTCAGGCGATTCTGGTCATT
IRE1	AGAGAGGCGGGAGAGCCGTG	CGAGGAGGTGGGGGAAGCGA
CHOP	TGGAAGCCTGGTATGAGGAC	TGTGACCTCTGCTGTTCTG
sXBP1	GCAGGTGCAGGCCAGTTGT	TGGGTCCAAGTTGTCCAGAATGC
ATF6	CCAGCAGCACCCAAGACTCAAACA	GTGTGACTCCCCCAGCAACAGC
<i>Mucin</i>		
MUC5AC	TCCACCATATACCGCCACAGA	TGGACGGACAGTCACTGTCAAC
<i>ABC transporters</i>		
MDR1	GCCAAAGCCAAAATATCAGC	TTCCAATGTGTTCCGGCAT
MRP1	GGGCTGCGGAAAGTCGT	AGCCCTTGATAGCCACGTG
MRP2	TGAGCAAGTTTGAAACGCACAT	AGCTCTTCTCCTGCCGTCTCT
BCRP	TGCAACATGTACTGGCGAAGA	TCTTCCACAAGCCCCAGG

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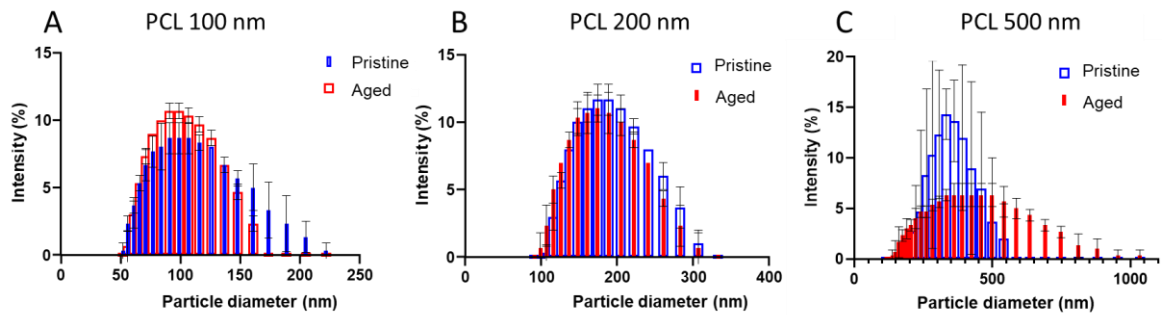
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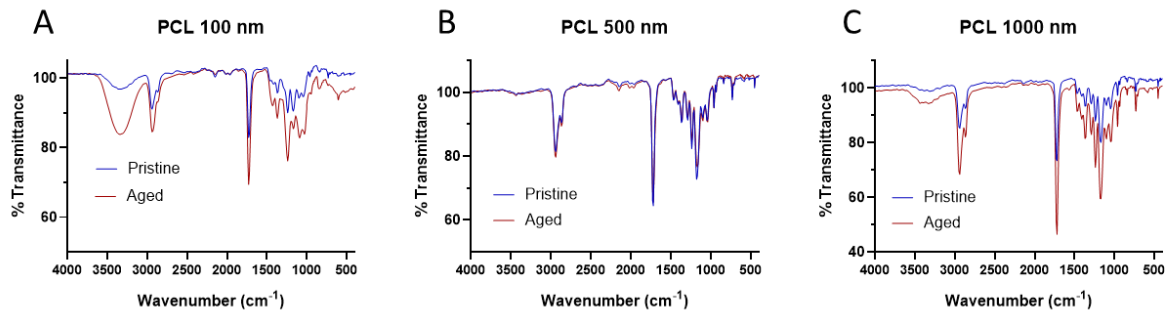
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1064 **Figure S1. Size distribution of PCL particles.** Size distribution histograms of pristine (blue) and aged (red) PCL  
 1065 particles, with primary diameter 100 nm (A), 200 nm (B) and 500 nm (C), as measured by DLS.

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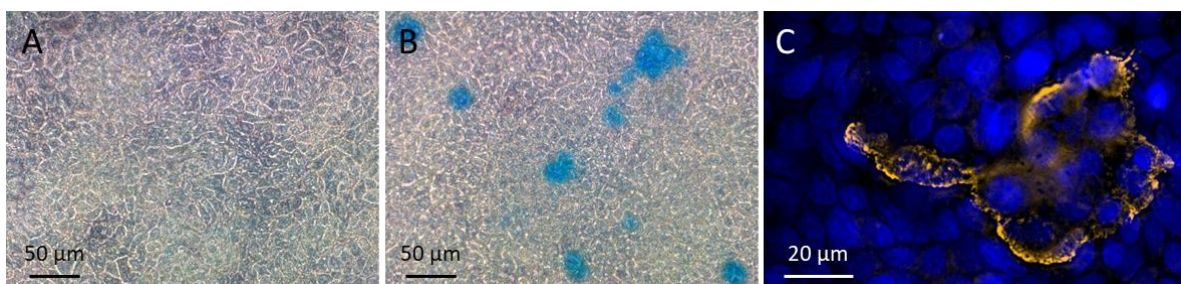
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1070 **Figure S2. FTIR analysis of PCL particles.** FTIR spectra of pristine (blue) and aged (red) PCL particles, with  
 1071 primary diameters 100 nm (A), 500 nm (B) and 1000 nm (C).

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1076 **Figure S3. Mucus secretion by Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX cells.** Alcian blue staining of Caco-2 NOD2<sup>1007fs</sup>  
 1077 monoculture (A) and Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX co-culture (B). MUC5AC immunostaining of the Caco-2  
 1078 NOD2<sup>1007fs</sup>/HT29-MTX co-culture (C).

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1080 **Table S2. Exposure metrics<sup>a</sup>**

**Nr. Particle per well**

*96-well*

Pristine	12,5 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
100 nm	2.9x10 <sup>11</sup>	5.9x10 <sup>11</sup>	1.2x10 <sup>12</sup>	2.4x10 <sup>12</sup>	4.7x10 <sup>12</sup>
200 nm	4.4x10 <sup>10</sup>	8.8x10 <sup>10</sup>	1.8x10 <sup>11</sup>	3.5x10 <sup>11</sup>	7.0x10 <sup>11</sup>
500 nm	6.4x10 <sup>9</sup>	1.3x10 <sup>10</sup>	2.5x10 <sup>10</sup>	5.1x10 <sup>10</sup>	1.0x10 <sup>11</sup>
1000 nm	1.4x10 <sup>10</sup>	2.7x10 <sup>10</sup>	5.4x10 <sup>10</sup>	1.1x10 <sup>11</sup>	2.2x10 <sup>11</sup>

*12-well*

Pristine	50 µg/mL
100 nm	1,2x10 <sup>13</sup>
200 nm	1,8x10 <sup>12</sup>
500 nm	2,5x10 <sup>11</sup>
1000 nm	5,4x10 <sup>11</sup>

Aged	12,5 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
100 nm	3.0x10 <sup>11</sup>	5.9x10 <sup>11</sup>	1.2x10 <sup>12</sup>	2.4x10 <sup>12</sup>	4.8x10 <sup>12</sup>
200 nm	5.2x10 <sup>10</sup>	1.0x10 <sup>11</sup>	2.1x10 <sup>11</sup>	4.2x10 <sup>11</sup>	8.4x10 <sup>11</sup>
500 nm	8.6x10 <sup>09</sup>	1.7x10 <sup>10</sup>	3.5x10 <sup>10</sup>	6.9x10 <sup>10</sup>	1.4x10 <sup>11</sup>
1000 nm	5.0x10 <sup>10</sup>	9.9x10 <sup>10</sup>	2.0x10 <sup>11</sup>	4.0x10 <sup>11</sup>	7.9x10 <sup>11</sup>

Aged	50 µg/mL
100 nm	1,2x10 <sup>13</sup>
200 nm	2,1x10 <sup>12</sup>
500 nm	3,5x10 <sup>11</sup>
1000 nm	2,0x10 <sup>12</sup>

**Million particle per cell**

*96-well*

Pristine	12,5 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
100 nm	11.8	23.5	47.1	94.1	188.2
200 nm	1.8	3.5	7.0	14.0	28.0
500 nm	0.3	0.5	1.0	2.0	4.1
1000 nm	0.5	1.1	2.2	4.3	8.6

*12-well*

Pristine	50 µg/mL
100 nm	39.2
200 nm	5.8
500 nm	0.8
1000 nm	1.8

Aged	12,5 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
100 nm	11.9	23.8	47,5	95.1	190.2
200 nm	2.1	4.2	8,4	16.8	33.6
500 nm	0.3	0.7	1,4	2.8	5.5
1000 nm	2.0	4.0	7,9	15.9	31.7

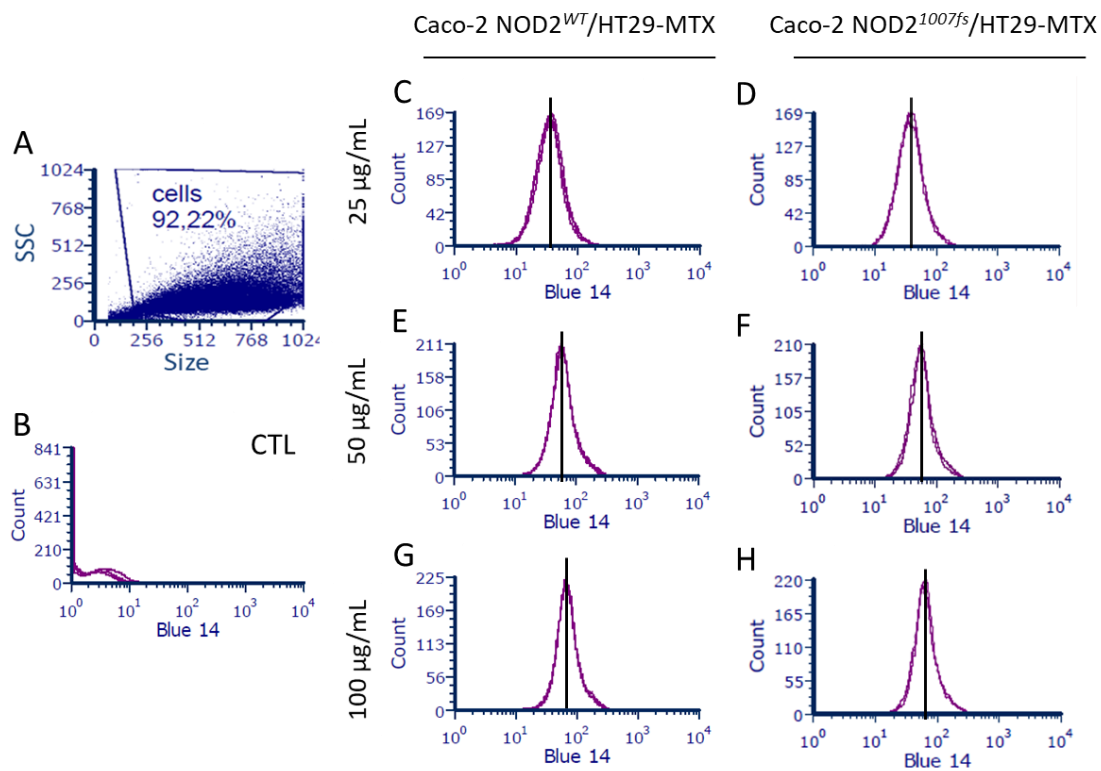
Aged	50 µg/mL
100 nm	39.6
200 nm	7.0
500 nm	1.2
1000 nm	6.6

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1082 <sup>a</sup>Particle number (Nr.) was estimated based on size distributions obtained by DLS, considering the % of particle  
 1083 from each size as reported by the zetasizer, the density of PCL particles as reported by the supplier, i.e., 1.145  
 1084 g/cm<sup>3</sup>, the volume of exposure medium in each well type (100 µL in 96-well plates and 1 mL in 12-well plates),  
 1085 and the particle mass concentration used in the considered assay (12.5, 25, 50, 100 and 200 µg/mL in 96-well  
 1086 plates for cytotoxicity experiments; 12.5, 25 and 50 µg/mL in 96-well plates for DHR123 assay; 50 µg/mL in 12-  
 1087 well plates for DNA damage assay, IL-8 secretion ELISA assay and RT-qPCR).

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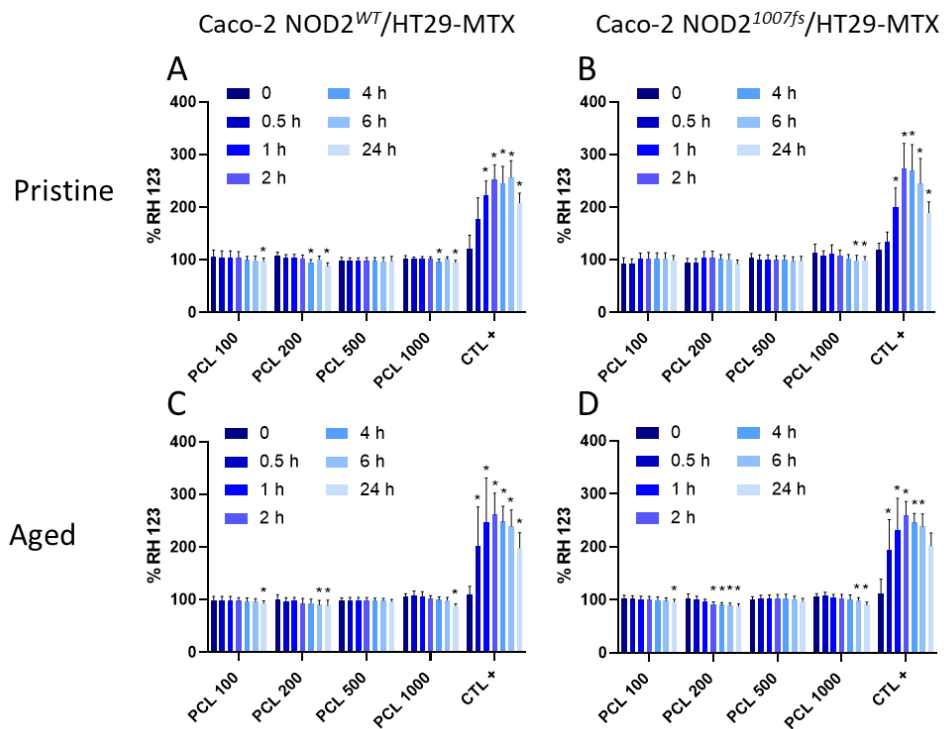
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1091 **Figure S4. Intracellular accumulation of PCL-DB14 particles, analysed by flow cytometry.** (A) gated cells, (B)  
 1092 fluorescence of control (unexposed) cells. (C-H) fluorescence of cells exposed to 25 µg/mL (C-D), 50 µg/mL (E-  
 1093 F), 100 µg/mL (G-H) of PCL-DB14, Caco-2 NOD2<sup>WT</sup>/HT29-MTX (B, C, E, G) and Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX (D,  
 1094 F, H).

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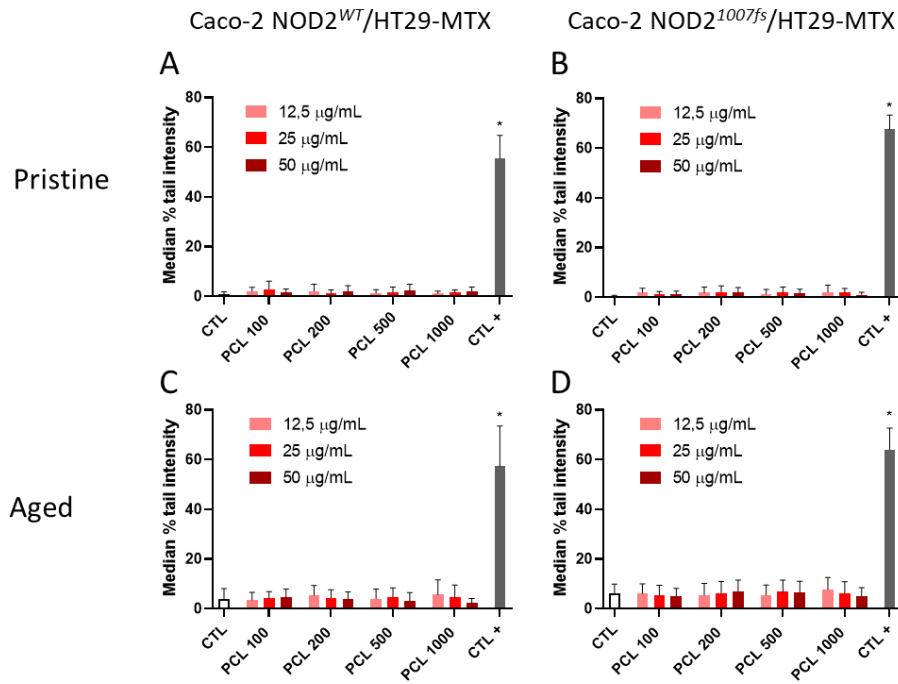


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1099 **Figure S5. Intracellular ROS level monitored throughout exposure time, in non-differentiated Caco-2 NOD2<sup>WT</sup>**  
 1100 **or 1007fs/HT29-MTX cells.** Cells were exposed 24 h after seeding, for 24 h, to 50 µg/mL pristine (A, B) or aged (C, D)  
 1101 PCL particles. Caco-2 NOD2<sup>WT</sup>/HT29-MTX (A, C) and Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX (B, D) cells. TBHP (1 mM) was  
 1102 used as positive control. Measurements were recorded immediately after exposure (0), or 0.5 h, 1 h, 2 h, 4 h, 6  
 1103 h or 24 h post-exposure. Results are expressed as % relative to the control (unexposed cells). Mean ± standard  
 1104 deviation; statistical significance: \*p<0.05, exposed vs CTL.

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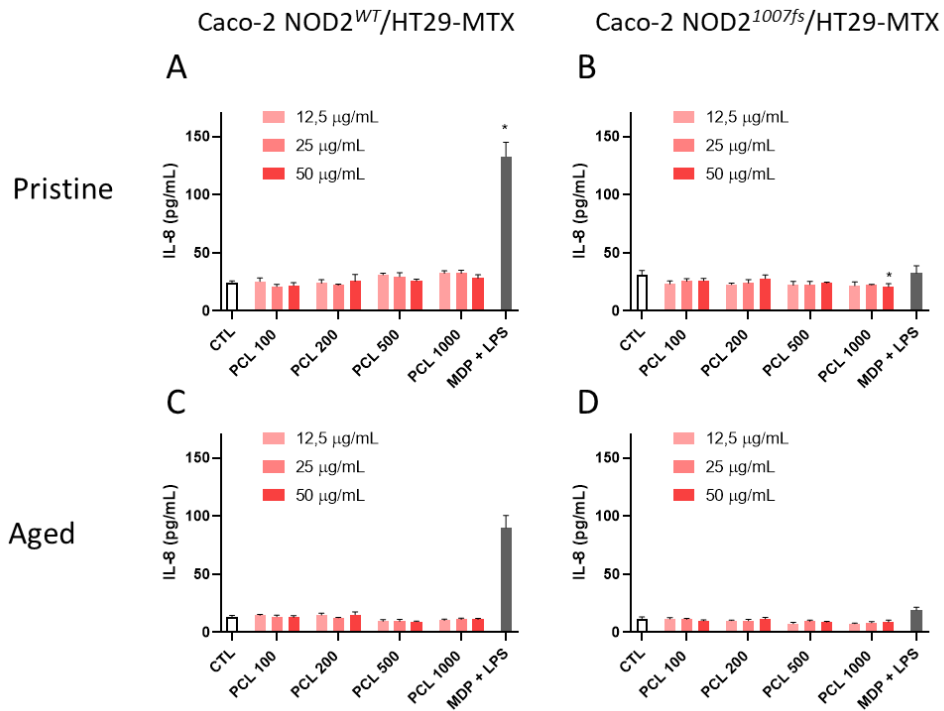
1108 **Figure S6. Genotoxicity of PCL particles towards non-differentiated Caco-2 NOD2<sup>WT</sup> or 1007fs/HT29-MTX cells.**

1109 Genotoxicity was assessed by the comet assay in alkaline conditions (no Fpg) in cells exposed for 24 h to 12.5  
 1110 µg/mL, 25 µg/mL or 50 µg/mL of pristine (A, B) or aged (C, D) PCL NPLs. Caco-2 NOD2<sup>WT</sup>/HT29-MTX (A, C) and  
 1111 Caco-2 NOD2<sup>1007fs</sup>/HT29-MTX (B, D) cells, exposed 24 h post-seeding. MMS (30 µg/mL) was used as positive  
 1112 control. Results are expressed as %Tail DNA. Mean ± standard deviation; statistical significance: \*p<0.05, exposed  
 1113 vs CTL.

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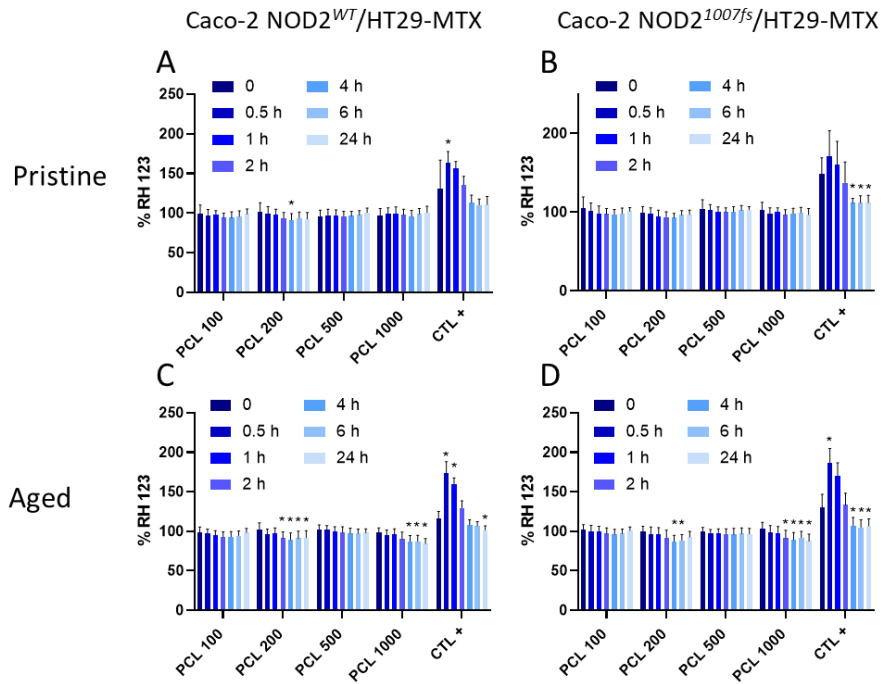
1118 **Figure S7. IL-8 secretion by non-differentiated Caco-2 NOD2<sup>WT</sup> or 1007fs/HT29-MTX cells after exposure to PCL**  
 1119 **NPLs.** IL-8 secretion was assessed using ELISA, in the exposure medium of cells exposed for 24 h to 12.5 μg/mL,  
 1120 25 μg/mL or 50 μg/mL of pristine (A, B) or aged (C, D) PCL NPLs. Caco-2 NOD2<sup>WT</sup>/HT29-MTX (A, C) and Caco-2  
 1121 NOD2<sup>1007fs</sup>/HT29-MTX (B, D) cells, exposed 24 h post-seeding. A mixture of MDP (10 μg/mL) and LPS (2 μg/mL)  
 1122 was used as a positive control. Mean ± standard deviation; statistical significance: \*p<0.05, exposed vs CTL.

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1128 **Figure S8. Intracellular ROS level monitored throughout exposure time, in differentiated Caco-2 NOD2<sup>WT</sup> or**  
 1129 **1007fs/HT29-MTX cells.** Cells were differentiated 21 days post-confluence, then, they were exposed for 24 h to 50  
 1130  $\mu\text{g/mL}$  pristine (A, B) or aged (C, D) PCL particles. Caco-2 NOD2<sup>WT</sup>/HT29-MTX (A, C) and Caco-2 NOD2<sup>1007fs</sup>/HT29-  
 1131 MTX (B, D) cells. TBHP (1 mM) was used as positive control. Measurements were recorded immediately after  
 1132 exposure (0), or 0.5 h, 1 h, 2 h, 4 h, 6 h or 24 h post-exposure. Results are expressed as % relative to the control  
 1133 (unexposed cells). Mean  $\pm$  standard deviation; statistical significance: \* $p < 0.05$ , exposed vs CTL.

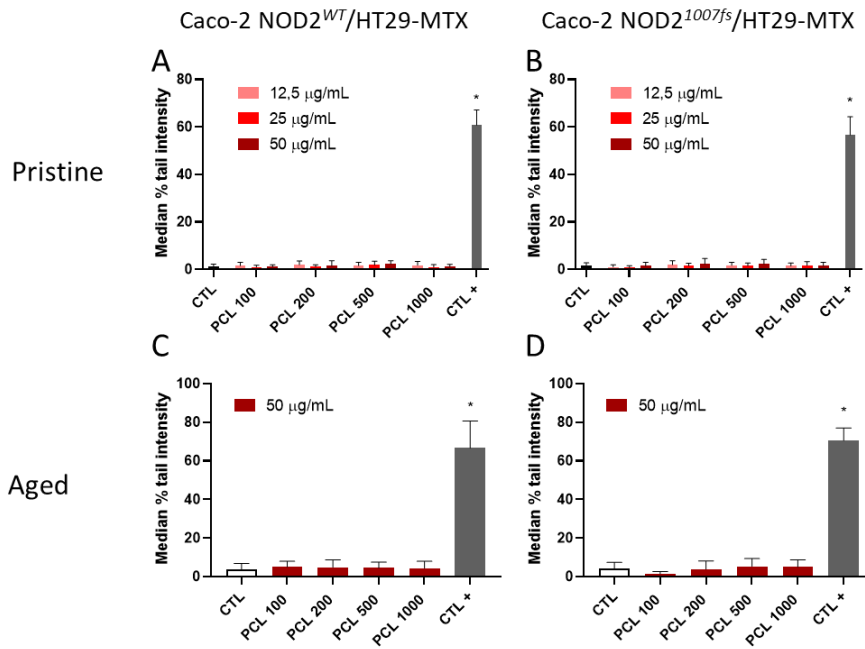
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1140 **Figure S9. Genotoxicity of PCL particles towards differentiated Caco-2 NOD2<sup>WT</sup> or 1007fs/HT29-MTX cells.** Cells  
 1141 were differentiated by growing them for 21 days post-confluence, before being exposed. Genotoxicity was  
 1142 assessed by the comet assay in alkaline conditions (no Fpg) in cells exposed for 24 h to 12.5 μg/mL, 25 μg/mL or  
 1143 50 μg/mL of pristine (A, B) or aged (C, D) PCL NPLs. Caco-2 NOD2<sup>WT</sup>/HT29-MTX (A, C) and Caco-2 NOD2<sup>1007fs</sup>/HT29-  
 1144 MTX (B, D) cells. MMS (30 μg/mL) was used as positive control. Results are expressed as %Tail DNA. Mean ±  
 1145 standard deviation; statistical significance: \*p<0.05, exposed vs CTL.

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