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Effect of Surface Modification on the Pulmonary and Systemic Toxicity of Cellulose Nanofibrils

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ABSTRACT
Cellulose nanofibrils (CNFs) have emerged as sustainable options for a wide range of applications. However, the high aspect ratio and biopersistence of CNFs raise concerns about potential health effects. Here, we evaluated the in vivo pulmonary and systemic toxicity of unmodified (U-CN), carboxymethylated (C-CN) and TEMPO (2,2,6,6-tetramethyl-piperidin-1-oxyl) oxidized (T-CN) CNFs, fibrillated in the same way and administrated to mice by repeated (3x) pharyngeal aspiration (14, 28 and 56 µg/mouse/aspiration). Toxic effects were assessed up to 90 days after the last administration. Some mice were treated with T-CN samples spiked with lipopolysaccharide (LPS; 0.02-50 ng/mouse/aspiration) to assess the role of endotoxin contamination. The CNFs induced an acute inflammatory reaction that subsided within 90 days, except for T-CN. At 90 days post-administration, an increased DNA damage was observed in bronchoalveolar lavage and hepatic cells in exposure to T-CN and C-CN, respectively. Besides, LPS contamination dose-dependently increased the hepatic genotoxic effects of T-CN.

Keywords: cellulose nanofibrils, fibrillated celluloses, functionalization, surface chemistry, pulmonary toxicity, genotoxicity

INTRODUCTION

Cellulose nanomaterials have raised great expectations in the last years as sustainable and environmentally friendly alternatives to mineral or synthetic counterparts. Their unique properties, such as high mechanical strength and elastic modulus, gas barrier properties, shear thinning behavior, and low thermal expansion allow their use for a broad range of applications. As such, cellulose nanomaterials are being applied in packaging, composites, emulsions, foams, electronics, cosmetics, medical devices and tissue engineering scaffolds, among others.
Cellulose nanofibrils are produced from wood and other sources by mechanical deconstruction of cellulose fibers into smaller fibrils through a fibrillation process. Chemical pre-treatments are usually applied prior to fibrillation, to ease deconstruction into homogeneous fibrils dispersions and to confer the nanofibrils with specific properties for different applications. TEMPO (2,2,6,6-tetramethyl-piperidin-1-oxyl) mediated oxidation and carboxymethylation are among the most frequently applied surface modifications to introduce negative charges onto cellulose fiber surfaces. It is worth noting, as no fibrillation process is totally efficient, that the resulting dispersions contain not only fibrils with sizes in the nanoscale but also larger fibrils (microfibrils). Moreover, surface modification not only affects the fibrillation yield and surface charge of the fibrils, but also other properties like their dimensions, colloidal stability and specific surface area. TEMPO-mediated oxidation is known to provide homogeneous aqueous dispersions. Furthermore, in addition to cellulose, other wood cell wall components, such as hemicelluloses and lignin, can also be present in small amounts in the dispersions of cellulose fibrils. For the sake of simplicity, herein we will use the term “cellulose nanofibrils” (CNFs) to generally refer to fibrillated celluloses.

The increasing commercial use of CNFs requires that the safety of these materials for human health and the environment is ensured. The fibrous nature of CNF, together with the reported high biopersistence, raise concerns about the potential health effects that nanofibrils could cause, especially if inhaled. Inhalation is the main exposure route in occupational settings, where workers may be exposed to airborne CNFs, e.g., during drying or spraying processes, or when handling dry materials.

Although an increasing number of studies have addressed the toxicity of cellulose nanomaterials in the last few years, reports on potential adverse health effects of CNFs are still scarce.
Moreover, the small set of reports available reveals conflicting results for CNF-induced toxic effects, which is partly due to the broad range of nanomaterials types and properties, arising from the fiber source and the production method, which can modulate toxic response to CNF. For instance, surface chemistry strongly determines how CNF interacts with their environment, dictating the colloidal stability and rheological and interfacial properties. The potential toxicological effects of CNFs that only differ in surface chemistry has thus far been evaluated by a few in vitro studies. Although in vitro models are appropriate for identifying acute effects and elucidating some primary mechanisms of action, they do not provide information on the behavior of the materials in complex systems, such as whole organisms. In vivo studies, on the other hand, allow the detection of health effects on a longer time span than cell culture-based assays, and can reveal secondary mechanisms of action.

To date, only a few in vivo studies have assessed the pulmonary effects of CNF. All of them were performed by exposing animals through (oro)pharyngeal aspiration or intratracheal instillation, which have been reported to be reliable methods for assessing the pulmonary outcomes of fibrous materials. These studies only concerned acute or sub-acute effects (up to 28 days post-exposure) and involved CNFs from different sources or produced by different methods. Therefore, comparisons among different CNF types have been challenged by the many variables affecting the results. A TEMPO-oxidized CNF (300–1000 nm in length, 10–25 nm in width) administrated by pharyngeal aspiration (10-200 µg/mouse) induced acute inflammatory response and increased DNA damage in the lungs of C57Bl/6 mice at 24 h post-administration. Four different CNFs manufactured by two different companies - two enzymatically pre-treated CNFs (2-20 µm in length, 2-20 nm in width), one carboxylated CNF (0.5-10 µm in length, 4-10 nm in width) and one carboxymethylated CNF (5-50 µm in length, 3-10 nm in width), together
with the bulk-sized material - were assessed for their immunogenic and genotoxic potential using the same animal model and dose range.\textsuperscript{14, 15} In addition, one of the enzymatically pre-treated CNFs and the carboxylated CNF were also assessed in C57Bl/6 mice intratracheally instilled with 6 and 18 µg/mouse.\textsuperscript{22} The enzymatically pre-treated CNFs were more prone to trigger inflammation than those modified by carboxymethylation or carboxylation, although the inflammatory response subsided within a month.\textsuperscript{15} Besides, carboxylation reduced systemic acute phase response.\textsuperscript{22} The enzymatically pre-treated CNFs, as well as the bulk fibers, induced more DNA damage in the lung than carboxylated and carboxymethylated CNFs, the damage being still observed 28 days after the administration. The carboxymethylated CNF and both enzymatic CNFs also showed significant DNA damage in bronchoalveolar lavage (BAL) fluid.\textsuperscript{14} Increased DNA damage was also observed in the lung tissue and BAL fluid of mice 28 days after intratracheal instillation of the enzymatic and the carboxylated CNFs, respectively.\textsuperscript{22} Another study performed with BALB/c mice exposed by aspiration to 40 and 80 µg/mouse of an unmodified CNF (142±14 nm in length, 56±14 nm in width) showed that the material induced a differentiation of T-cells toward a Th1-phenotype at 14 days post-exposure.\textsuperscript{21}

The generally reported inflammogenic response following pulmonary exposure to CNF may have been induced by the endotoxins carried by the nanofibrils. Endotoxins (\textit{e.g.}, lipopolysaccharide, LPS), bacterial components frequency found as contaminants in cellulosic materials,\textsuperscript{24} can trigger toxicological responses when administrated to animals.\textsuperscript{25, 26} As CNFs are typically produced in the absence of aseptic conditions, contamination with endotoxins may occur at any step of the manufacturing process.\textsuperscript{26} Therefore, CNFs are usually sterilized by autoclaving or adding biocides before testing. However, it is unclear whether these treatments could affect the toxicological properties of CNFs.
In the present study, we evaluated the *in vivo* pulmonary and systemic toxicity of unmodified cellulose nanofibrils (U-CNФ) and two functionalized CNFs, carboxymethylated (C-CNФ) and TEMPO-oxidized (T-CNФ). The CNFs were obtained from the same source, fibrillated in the same way, and tested as produced without further treatments. CNFs were administrated to mice by repeated pharyngeal aspiration, and the inflammatory and genotoxic effects were assessed up to 90 days after the last administration. In parallel, T-CNФ samples spiked with increasing amounts of LPS (0.02-50 ng/mouse/aspiration) were included to assess the potential role of endotoxin contamination. Our findings indicate that all CNFs induced an acute inflammatory reaction that mostly subsided within 90 days, even though CNФ was still present in the lungs at that time point. Pulmonary exposure to anionic modified CNFs was associated with local or systemic genotoxic effects at 90 days post-administration. On the other hand, LPS contamination modulated the hepatic response to CNФ at the same time point.

**EXPERIMENTAL SECTION**

**Synthesis and surface modification of CNFs.** The CNFs were tested as produced, without further treatments (*e.g.*, sterilization) that may modify their properties.27 As the processing was performed in a non-sterile bench-scale laboratory, special care was taken during the production processes to prevent bacteria contamination. The surface of all the used equipment was extensively cleaned through consecutive washes with deionized water, ethanol (Altia, Helsinki, Finland), sterile purified water, and endotoxin-free water (Thermo Fisher Scientific, UT, USA). Sterile centrifuge tubes (Thermo Fisher Scientific, UT, USA) and sterile and endotoxin-free water were also used to prepare the samples in aqueous media.
CNFs were sourced as a commercial low endotoxin containing bleached sulfite birch dissolving pulp (UPM Kymmene Oyj, Finland) with a lignin content below 0.5%. The wood fibers were refined using a laboratory-scale PFI refiner (Hamjern Maskin AB, Norway). The refined fibers were then used as such (unmodified) or treated by TEMPO-(2,2,6,6-tetramethyl-piperidin-1-oxyl) oxidation, or carboxymethylation.

The TEMPO oxidation procedure has been fully explained by Imani et al.\textsuperscript{28, 29}. Briefly, 2 g of the refined fibres (base on dry weight) were treated by sodium bromide (NaBr) and TEMPO oxidation (1.0 mmol.g\textsuperscript{−1}, and 0.1 mmol.g\textsuperscript{−1}, respectively) after dispersion in distilled water (200 cm\textsuperscript{3}, 1\% (w/v) solid contents). Following the above procedures, the resulting fibre suspensions were stirred for 5 min at 700 min\textsuperscript{−1} (rpm) using an Ultra Turrax mixer, (IKA, T 25 digital). Subsequently, 10\% NaClO solution was adjusted to pH 10 using a dosage level of 1.2 mmol.g\textsuperscript{−1} based on fibre dry weight then 0.5 M NaOH solution was added dropwise into the dispersion to maintain the pH at 10. Finally, after subjecting the fibres to TEMPO- mediated oxidation for 3 h, they were thoroughly washed with distilled water to 1.5 \% (w/v) solid contents, followed by passage through a microfluidiser for 6 times (Microfluidics, M-110 P, Massachusetts, USA). The mild oxidation conditions used in our TEMPO treatment were chosen to produce low charge density fibrillated cellulose, thus minimizing the morphological differences compared with the carboxymethylated fibrils. In this way, our comparisons emphasize the effect of modification types.

The preparation of the carboxymethylated fibers was performed according to the method detailed by Im et al.\textsuperscript{30}. In brief, 1 g (dry weight) of the refined fibers were solvent exchanged to ethanol (three times washing in 100 ml ethanol), then concentrated by pressure filtration to a solid
content of 18 wt% and placed in a round-bottom flask. Following, the fibers were impregnated with 0.96 mmol.g\(^{-1}\) of monochloroacetic acid in 200 mL isopropanol for 30 min at 35 °C. Then, the fibers were added in a solution of 3.68 mmol.g\(^{-1}\) of NaOH dissolved in a 300 mL at a volume ratio of 1:4 mixture containing methanol and isopropanol, respectively. The pretreated fibers were washed with water and filtered until pH and conductivity were reached to 7.0±0.5 and of≤20 µS/cm, respectively.

The unmodified and pretreated fibers were diluted to 1.5% solids content, and nanofibrils were obtained by disintegration through microfluidization after six cycles (Microfluidics, M-110 P, Massachusetts, USA), as previously reported.\textsuperscript{28, 29} In this way, three different types of CNF were obtained: unmodified (U-CNФ), TEMPO oxidized (T-CNФ) and carboxymethylated (C-CNФ). The resulting aqueous dispersions were concentrated by slow evaporation in an oven until reaching a 1.0-1.5% concentration, which was considered adequate for toxicity testing.

**Characterization of the CNFs.** Atomic force microscopy (AFM) was used to investigate the morphology of the CNF samples using a Dimension 5000 scanning probe microscope (Veeco, TX, USA). The measurements were performed in tapping mode by a Veeco Nanoscope with V controller (Veeco) in air using MicroMash silicon cantilevers (NSC15/AIBS). Samples were prepared by placing the respective CNF aqueous dispersion (0.001 wt %) in an ultrasonic bath (240 W, 50/60 Hz; BANDELIN, Germany) for 30 min. A droplet of the CNF aqueous dispersion was cast onto a microscope glass slide and allowed to dry at room temperature for 24 h.

Scanning electron microscopy (SEM) analyses were carried out using a field emission gun scanning electron microscopy (FEG-SEM) microscope (Zeiss Sigma VP, Jene, Germany) at an acceleration voltage of 1.5 kV with a field emission gun. For this purpose, the CNF aqueous
dispersions were freeze-dried (0.001 wt. %) overnight and gold-sputtered to a thickness of 5 nm. The images obtained from the AFM and SEM analyses were subjected to ImageJ (USA) analysis by using 100 nanofibrils from each CNF sample, as recommended by Foster et al.,
yielding the respective size distribution profile.

To evaluate the yield of the production of the nanofibrils, a 0.2 wt% cellulose fibril suspension (40 mL) was centrifuged at 9000 rpm for 30 min. The percentage of supernatant material was considered as the yield of fibrillation.

X-ray diffraction (Model X’Pert PRO, Philips PANalytical, Netherlands) spectra were obtained to determine the crystallinity of the freeze-dried CNFs. The samples were scanned in the range of 2θ = 5–50° using a scanning rate of 0.5° min⁻¹ at 45 kV voltage and 40 mA electric current.

The electrostatic charge was assessed by using ζ-potential measurements. Dispersions of 0.001 % (w/w) of the CNF samples were prepared in water through ultrasonication for 60 s using a universal dip cell in a ZetaSizer Nano instrument (Nano ZS, Malvern Instruments Ltd, Worcestershire, UK). The measurements were repeated three times for each sample at 25 °C.

The concentration of carboxyl groups (COOH) was determined by conductivity titration. Briefly, a dried sample (∼50 mg) was mixed with deionized water (20 mL) and 0.01 M HCl (15 mL), and the mixture was stirred while the pH was set to 3.0 with HCl. Then, a 0.01 M NaOH solution was added until pH 11. The carboxylate content of the samples was determined from the sudden change in conductivity. To determine the aldehyde content (CHO), a suspension of 10% cellulose/water mixture (20 g) was mixed with deionized water (100 mL), adjusted to pH 4. An excess amount of 5% w/w hydroxylamine hydrochloride solution was added to the sample, set to
pH 4 using 0.05 m NaOH and allowed to react for 2 h. The concentration of aldehydes in the sample was calculated from the moles of NaOH consumed to reach pH 4.

Bacterial lipopolysaccharide content (endotoxin levels) was measured using the Pierce™ Chromogenic Endotoxin Quant Kit (Thermo Fisher Scientific, Waltham, MA, USA), which has no interference from β-glucans, following the instructions provided by the manufacturer. Prior to endotoxin test, all samples were heated at 75 °C for 15 min, to promote the release of endotoxins from the material as previously described.²⁶

**CNF Dispersion.** Stock dispersions (2 mg mL⁻¹) were prepared in endotoxin-free water by diluting the CNF aqueous dispersions, and then mixed vigorously by high speed vortexing for 20 s, as recommended by Bitounis *et al.*³² Then, serial dilutions were prepared in water, and mixed by vortexing for 20 s immediately before being administrated to mice.

**Animals.** Female C57BL/6 mice (7-8 weeks old, average weight 20 g) were purchased from Scanbur AB (Sollentuna, Sweden) and quarantined for 1 week. The mice were randomly assigned to groups of 3-4 animals/cage and housed in ventilated plastic cages bedded with aspen chip. The animals were provided with standard mouse chow diet and tap water *ad libitum*. The environment of the animal room was carefully controlled, with a 12-h dark/light cycle, temperature of 20-21 °C, and relative humidity of 40-45 %. The mice were weighed at the beginning and in the end of the experiment, and their health was carefully monitored throughout the experiment. The experiments were performed in agreement with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg March 18, 1986, adopted in Finland on May 31, 1990). The study was approved by
the Animal Experiment Board and the State Provincial Office of Southern Finland (license number ESAVI/31843/2019).

**Pharyngeal aspiration exposure.** Pharyngeal aspiration exposure was performed as previously described. Three (1 day post-exposure) or six (28 and 90 days post-exposure) mice per group were exposed to repeated (3x) doses (50 µL/mouse) of each CNF at 14, 28 and 56 µg/mouse/aspiration, resulting in accumulative doses of 42, 84 and 168 µg/mouse. This range of doses corresponded to the ones tested in previous studies with nanocellulose materials. In addition, it covered realistic levels of human exposure as, in the case of nanocrystalline cellulose, an accumulative dose of 240 µg/mouse was reported to be equivalent to the dose of a human worker exposed for 42 working days to the U.S. Occupational Safety and Health Administration’s (OSHA) 5 mg m$^{-3}$ permissible exposure limit for the respirable fraction of cellulose dust. On the other hand, after assessment of nanocrystalline cellulose’s facilities, the maximum estimated concentration of detected airborne cellulose was more than ten times below the OSHA limit. Vehicle control mice received 50 µL/aspiration of endotoxin-free water. A positive control group was included for the inflammatory response and the induction of micronuclei. Within this group, each animal received a pharyngeal aspiration of 28 µg multiwalled carbon nanotubes (MWCNTs; MWCNT-XNRI-7 from Mitsui & co., Ltd., Tokyo, Japan) and, at 28 and 90 days post-exposure, also a single dose of 40 µg of mitomycin C (MMC, Sigma–Aldrich, Steinheim, Germany) intraperitoneally injected 48 h before euthanizing the animals. MWCNTs were included due to their capacity to induce acute and sub-acute pulmonary inflammation in mice, and were dispersed as previously described. MMC is a genotoxic compound recommended to be used as positive control in the *in vivo* micronucleus assay. The mice were euthanized by an overdose of isoflurane at 1, 28 and 90 days after the last exposure.
To assess the potential effects of endotoxin contamination, additional groups of mice were treated with T-CNФ samples (14 µg/mouse/aspiration) spiked with increasing amounts of LPS (0.02, 1 and 50 ng/mouse/aspiration). T-CNФ was chosen as the carrier of LPS, as this type of functionalization has been reported to render low levels of endotoxin contamination. To ensure a LPS-induced inflammatory response, 50 ng/mouse/aspiration, which has previously been reported to induce a strong acute inflammation in mice, was chosen as the highest dose. This dose corresponded to $10^4$ EU mL$^{-1}$ of LPS. The two lower doses (1 and 0.02 ng/mouse/aspiration) corresponded to 200 and 4 EU mL$^{-1}$.

For all the analyzed endpoints, the unmodified and surface-modified CNФ were compared with the vehicle group, whereas the LPS-spiked T-CNФ samples were compared with the corresponding T-CNФ treatment (the uncontaminated T-CNФ at 14 µg/mouse/aspiration).

**Sample collection.** Blood was collected from the *vena cava*, mixed with 5 % ethylene diaminetetraacetic acid (EDTA; Merck KGaA, Darmstadt, Germany) in a tube (to prevent coagulation) and stored on ice. The trachea was cannulated with a blunted 22-gauge needle, and bronchoalveolar lavage (BAL) was performed once with 800 µL of phosphate buffered saline (PBS; Lonza, Walkersville, MD, USA), to collect a BAL sample for the enumeration of inflammatory cells and transmission electron microscopy (TEM) analyses, and then infused four times with 800 µL of 0.15 M NaCl (Baxter Healthcare SA, Zurich, Switzerland), to collect a BAL sample for the comet analyses. Both BAL samples were stored on ice until being processed. The chest of the mouse was opened, and the right lung lobules were removed, and placed into a tube containing cold Merchant’s medium for the comet assay. Pieces of liver were processed in a similar way. The rest of the lung and pieces of liver were fixed in 10% formalin
for histopathological analyses, and a piece of the lung was fixed with 2.5 % glutaraldehyde and prepared for TEM analyses.

**BAL fluid cellularity.** One hundred µL of the first BAL sample was cytocentrifuged onto a microscope slide, air dried and stained with May-Grünwald-Giemsa (MGG; Reagena, Toivala, Finland). A minimum of 100 inflammatory cells (classified as macrophages, neutrophils, eosinophils, or lymphocytes) per animal were analyzed using a Zeiss Axioplan light microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany) at 40x magnification. Another aliquot (100 µL) was used to determine the total number of inflammatory cells using a flow cytometer (CytoFlex S, Beckman Coulter, Indianapolis, IN, US).

**Histological evaluation.** Tissue sections of lung and liver collected in 10 % formalin were fixed for 24 h at room temperature. The samples were thereafter embedded in paraffin, cut, affixed on slides, and stained with hematoxylin and eosin as previously described. The slides were examined with a light microscope (Zeiss Axioplan, Carl Zeiss Microscopy GmbH, Göttingen, Germany) and the scoring of different histological events were based on subjective semi-quantitative assessment, as previously described. The different events included in the analysis were: i) macrophage infiltrates (which refers to dense collections of >10 cells), ii) lymphocyte aggregates (which refers to dense collections of >20 cells), iii) neutrophilic aggregates (which refers to dense collections of >10 cells), iv) eosinophilia (an infiltration of eosinophils into the lung), v) free material in bronchia and alveolar space, and vi) granuloma (material surrounded by a dense macrophage layer).

**Lung biopersistence.** The presence of CNF and MWCNTs in the lung tissue was assessed at all time points by light microscopy in association with the histopathological evaluation. As light
microscopy only allows the detection of material aggregates, TEM was used to assess whether nanofibrils were located inside the alveolar or bronchial spaces or within cells at 90-days post-exposure. BAL and lung samples were fixed in 2.5 % glutaraldehyde at 4 °C for 24 h, and thereafter stored refrigerated in PBS. Then, samples were placed onto uncoated copper grids, post-fixed in osmium tetroxide, and stained with uranyl acetate and lead citrate, as described previously.\(^{41}\) Samples were analyzed using a Jeol JEM-1400 Flash TEM (Jeol Ltd, Tokyo, Japan) operated at an acceleration voltage of 80 kV and equipped with a Matataki Flash sCMOS camera (Jeol Ltd, Tokyo, Japan).

**Comet assay from BAL, lung, and liver suspensions.** DNA damage was assessed by the comet assay in mouse samples collected at 28- and 90-day post-exposure and processed as previously described.\(^{36}\) In brief, pieces of lung and liver were minced in chilled Merchant’s medium and mechanically dispersed into a single cell suspension using a cell strainer (40 µm Ø; VWR International LLC, Radnor, USA). A small proportion of the cell suspensions was exposed to hydrogen peroxide (H\(_2\)O\(_2\), 100 µM) \textit{ex vivo} and used as an internal positive control to verify the performance of the comet assay. The comet assay was performed in alkaline conditions (\(pH > 13\)) as described previously.\(^ {42}\) The slides were coded, and one scorer performed the comet analysis using a fluorescence microscope (Axioplan 2, Zeiss, Jena, Germany) and an interactive automated comet counter (Komet 5.5, Kinetic Imaging Ltd., Liverpool, UK). The percentage of DNA in comet tail was analyzed from two slides per animal (75 cells/slide, 150 cells/animal) to measure the amount of DNA damage.

**Micronucleus assay in peripheral blood erythrocytes.** Systemic chromosome damage was assessed by the micronucleus assay in mouse samples collected at 28- and 90-day post-exposure. Blood samples were diluted 1:5 in fetal bovine serum (Life Technologies Limited, Paisley, UK)
and smeared onto microscopical slides, dried at room temperature overnight and fixated in methanol (Merck KGaA, Darmstadt, Germany). The slides were stained with MGG, and the micronucleus analysis was performed in accordance with TG 474. Stained slides were analyzed using a light microscope (Zeiss Axioplan, Carl Zeiss Microscopy GmbH, Göttingen, Germany) at 40x magnification. Two thousand normochromatic erythrocytes (NCEs) per animal were scored for the frequency of micronucleated normochromatic erythrocytes (MNCEs). In addition, two thousand polychromatic erythrocytes (PCEs) per animal were scored in the vehicle and positive control group, for the frequency of micronucleated polychromatic erythrocytes (MPCEs). Besides, the ratio of PCEs to NCEs was assessed in 2000 erythrocytes per animal as an indicator of bone marrow toxicity.

**Statistical analyses.** Statistical analyses of the body weight, neutrophils, and other cell counts in BAL, cell aggregates/infiltrates in lungs, frequency of micronucleus and percentage of PCEs were done as described by Hadrup et al. Data were tested for normality with the Shapiro-Wilk test, and for homogeneity of variance with the F test or the Brown-Forsythe test (for two or more than two sample comparisons, respectively). In case of deviations in normality or in homogeneity of variance, the non-parametric Mann Whitney (two groups) or Kruskall–Wallis (more than two groups) tests were applied. Otherwise, differences were assessed by one-way t-test or one-way analysis of variance (ANOVA). In addition, Dunn’s and Dunnett’s multiple comparisons tests (Kruskall–Wallis test and ANOVA, respectively) were used for an *a posteriori* comparison of each of the doses with the corresponding zero control.

A hierarchic ANOVA was used to study, if the percentage of DNA in tail in BAL and lung cells was influenced by the treatments, as recommended by Bright *et al.* Bonferroni’s test was applied as *a posteriori* comparison among the means.
For all endpoints analyzed, dose–dependent relationships were investigated by linear regression analysis.

Differences were interpreted to be significant if \( p < 0.05 \). The analyses were performed using the IBM SPSS Statistics for Windows (2013), Version 22.0 program.

RESULTS

Characterization of the CNF samples. Figure S1 presents FEG-SEM images of the CNF samples at low magnification, showing the main structural and morphological features of the nanofibrils. They included large fibrils and fragments originated from the cell wall of fibers given the limited extend of fibrillation. Also, it is possible that fibril aggregates formed upon removal of water during the sample preparation. As shown in Table 1, T-CNDF reported the highest fibrillation yield (78%), followed by C-CNDF (69%) and U-CNDF (58%). In addition, our TEMPO oxidation conditions were rather mild, which led to fibrils of similar sizes compared to the carboxymethylated ones. No significant differences in the lateral fibril width were observed even though T-CNDF indicated smaller sizes (Table 1 and Figure 1 (b,e,h), as is also generally expected for this CNF grade.

The structural morphology and fibril diameter distributions of the CNF samples are shown in Figure 1. Besides, the average width and length of individual fibrils, as well as the aspect ratio, are shown in Table 1. The T-CNDF sample showed the lowest lateral dimension and length, with values in the range of 7.69±0.9 and 1589±220 nm, followed by the C-CNDF and U-CNDF samples. The aspect ratio of the nanofibrils varied from 175:1 (U-CNDF) to 214:1 (C-CNDF), although the observed differences between surface modified nanofibrils were small. All the samples showed a
good degree of colloidal dispersion in water, as judged by direct visual observation on vials containing the given aqueous dispersions.

**Figure 1.** Atomic force microscopy (AFM; top row) and scanning electron microscopy (SEM; middle row) images, and fibril lateral size distributions (fitted log-normal models; bottom row) of U-CNF (a,d,g), T-CNF (b,e,h), and C-CNF (c,f,i).

Table 1 also includes the degree of crystallinity, ζ-potential values, and content of functional groups of the CNF samples. The minimum and maximum degree of crystallinity of the CNF samples varied from 67 to 75% (for T-CNF and U-CNF, respectively), indicating that the
chemical modification did not considerably alter the crystallinity of the CNFs. It is known that the ζ-potential value tracks with the charge density, e.g., dissociated carboxylic groups on the surface of fibrils. As expected, all the CNF samples were anionic according to their ζ-potential. T-CNФ had the highest carboxyl group density, and a low aldehyde content (0.07 mmol.g⁻¹), comparable to that of C-CNФ. Low levels of carboxyl groups were quantified in U-NFC, which presented the lowest ζ-potential values. A good colloidal stability in aqueous media was observed for the suspension of modified fibrils.

Table 1. Physico-chemical characteristics and endotoxin levels of the fibrillated cellulose samples. Fiber diameter and length, and ζ-potential are expressed as mean±SD.

<table>
<thead>
<tr>
<th>Material</th>
<th>U-CNФ</th>
<th>T-CNФ</th>
<th>C-CNФ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface modification</td>
<td>None</td>
<td>TEMPO oxidation</td>
<td>Carboxymethylation</td>
</tr>
<tr>
<td>Fibrillation yield [%]</td>
<td>54</td>
<td>78</td>
<td>69</td>
</tr>
<tr>
<td>Degree of crystallinity [%]</td>
<td>75</td>
<td>67</td>
<td>71</td>
</tr>
<tr>
<td>Fibril width (D) [nm]⁽ᵃ⁾</td>
<td>11.5±0.07</td>
<td>7.7±0.90</td>
<td>8.5±0.04</td>
</tr>
<tr>
<td>Length (L) [nm]⁽ᵃ⁾</td>
<td>2011±301</td>
<td>1589±220</td>
<td>1820±119</td>
</tr>
<tr>
<td>Aspect ratio (L/D)</td>
<td>175</td>
<td>207</td>
<td>214</td>
</tr>
<tr>
<td>ζ-potential [mV]</td>
<td>-19±2</td>
<td>-27±3</td>
<td>-30±1</td>
</tr>
<tr>
<td>Carboxyl group content [mmol.g⁻¹]</td>
<td>0.02</td>
<td>0.41</td>
<td>0.35</td>
</tr>
<tr>
<td>Aldehyde group content [mmol.g⁻¹]</td>
<td>0.014</td>
<td>0.070</td>
<td>0.030</td>
</tr>
<tr>
<td>Endotoxin level [EU mL⁻¹]</td>
<td>&gt; 1.2⁽ᵇ⁾</td>
<td>0.22</td>
<td>0.15</td>
</tr>
</tbody>
</table>

⁽ᵃ⁾Determined by atomic force and scanning electron microscopy;⁽ᵇ⁾Measured by the Pierce™ Chromogenic Endotoxin Quant Kit;⁽ᶜ⁾Levels above the 0.5 EU mL⁻¹ limit value established by the US Food Drug Agency (FDA) for inhalation studies;⁽ᵈ⁾1.2 EU mL⁻¹ was the detection limit allowed by the kit.
Results from the endotoxin analyses are also shown in Table 1. Both surface modified CNFs had a low level of endotoxin contamination, below the 0.5 EU mL$^{-1}$ limit value established by the US Food Drug Agency for inhalation studies. On the other hand, U-CNFR showed a high endotoxin level that exceeded the detection limit of the kit (>1.2 EU mL$^{-1}$).

**Clinical signs and body weight.** No clinical signs of toxicity were observed during the study period.

A decrease in body weight was observed in mice exposed to the highest dose (56 µg/mouse/aspiration) of U-CNFR ($p < 0.01$) and to MWCNTs ($p < 0.0001$) at 1 day after the last administration (Figure S2). However, the effect was already reversed for both materials at 28 days post-exposure. No reduction in body weight gain was observed in mice exposed to increasing doses of LPS-spiked T-CNFR samples in comparison with the corresponding T-CNFR treatment (uncontaminated T-CNFR 14 µg/mouse/aspiration).

**Neutrophil counts in bronchoalveolar lavage fluid.** BAL fluid cell composition was determined on day 1, 28 and 90 after the last administration (Table S1). Figure 2 shows the results on inflammation, measured as neutrophil influx. All CNF samples induced an acute inflammatory reaction on day 1, which was not statistically significant in most of the cases, due to the small group size ($n = 3$). A statistically significant increase in neutrophil count was observed at the highest dose of T-CNFR and C-CNFR, and for the positive control. In addition, C-CNFR showed a significant increasing linear dose-response ($p = 0.0002$, slope = 463.7).
**Figure 2.** Total number of neutrophils in BAL fluid at 1 (A), 28 (B) and 90 (C) days of vehicle, CNF samples, positive control, and LPS-spiked T-CNF samples exposure. Data are expressed as means ± SEM. Asterisks designate statistically significant differences compared with the vehicle group at *p* < 0.05 and **p** < 0.01. The LPS-spiked T-CNF samples were compared with the corresponding T-CNF treatment (14 µg/mouse/aspiration) and no significant differences were found.

The initial strong neutrophil influx progressively subsided within 90 days except for T-CNF. At 28 days post-exposure, a significant increase in neutrophils was present for the middle dose of U-CNF (*p* = 0.0201), the two highest doses of T-CNF (*p* = 0.0223 and 0.0016, respectively) and the highest dose of C-CNF (*p* = 0.0012). In addition, a significant increasing dose-response was observed for the three CNF samples, U-CNF (*p* = 0.0053, slope = 15.05), T-CNF (*p* = 0.0027, slope = 31.80) and C-CNF (*p* = 0.0031, slope = 23.11).

At 90 days post-exposure, the total number of neutrophils was statistically significantly different from the vehicle level (*p* = 0.0124) for the highest dose of T-CNF (56 µg/mouse/aspiration), and similar to the number of neutrophils induced by the positive control. Furthermore, T-CNF induced a significant linear dose-response at this time point (*p* = 0.0004, slope = 28.5). U-CNF
also induced a statistically significant increasing dose-response ($p = 0.0148$, slope = 14.81), although none of the doses differed from the vehicle group.

Statistically significant increases in the BAL eosinophilic population were observed in mice exposed to T-CNF (1 d post-exposure), and to U-CNF and the positive control (28 d post-exposure). Increased eosinophil influx was induced by none of the materials at 90 d post-exposure (Table S1).

The LPS-spiked samples showed a similar behavior as the corresponding uncontaminated T-CNF at 14 µg/mouse/aspiration (Figure 2). After the strong neutrophil influx at 1-day post-exposure, the number of neutrophils dropped at 28- and 90-day post-exposure. Nor significant linear regression was observed at any of the time points. No significant increased eosinophil influx was observed at any time point (Table S1).

**Histopathology evaluation.** Recorded histopathological changes are presented in Tables S2-S4. In addition, representative images of the most predominant histological changes at each studied timepoint are presented in Figure 3. Vehicle-exposed mice initially showed some inflammatory changes that were resolved at 28- and 90-days post-exposure (Figure 3A). Overall, the differences among the CNF-treated mice samples were minor. At 1-d post-exposure, all CNF materials induced an acute peri-bronchial neutrophilic inflammatory reaction accompanied by eosinophilia (Table S2), as illustrated in Figure 3B. As eosinophilia was recorded as the incidence of animals showing this effect, the results do not allow quantification of the reaction. In addition, macrophage infiltrates were seen in the alveolar region around material aggregates. The initial reaction had mostly resolved 28 days post-exposure (Table S3 and Figure 3C), but mild dose-dependent peri-bronchial neutrophilic reaction and some macrophage infiltrates
persisted even at 90 days post-exposure (Table S4 and Figure 3E). Eosinophilic crystals were observed in animals exposed to any type of CNF at 90 days post-exposure (Figure 3D), although no clear incidence of eosinophilia was recorded at this timepoint (Table S4).

**Figure 3.** Representative images of hematoxylin- and eosin-stained lung sections of mice exposed to the vehicle (A), CNF (B-E, 28 µg/mouse/aspiration) or the positive control (F). CNF aggregates are marked with black arrows. (A) Vehicle sample on day 90. (B and inset) Neutrophil influx accompanied by eosinophils in a T-CN sample 1-day post-exposure. (C) Material in lung tissue in a T-CN sample 28 days post-exposure. (D and inset) Eosinophilic crystals in a C-CN sample 90 days post-exposure. (D) Material in lung tissue in a U-CN sample
sample 90 days post-exposure. (F) Granuloma formation around material aggregates in a positive control sample 90 days post-exposure.

The LPS-spiked T-CNF samples induced a slightly stronger neutrophilic inflammatory reaction than the corresponding uncontaminated material (T-CNF, 14 µg/mouse/aspiration) 1-day post-exposure (Table S2), but histopathological changes at 28- and 90-days post-exposure were comparable to the uncontaminated T-CNF sample (Table S3 and S4).

CNF induced a weaker pulmonary inflammation than the positive control treatment, which included long, straight MWCNTs (28 µg/mouse/aspiration). The positive control produced a strong acute peri-bronchial neutrophilic reaction and a higher number of macrophage infiltrates in the parenchyma than any of the CNF materials (Tables S2). The inflammatory reaction persisted 28 days after the administration (Table S3). In addition, positive control mice, but not CNF-treated ones, showed signs of granuloma formation in the lung tissue 90 days after the pulmonary exposure (Table S4 and Figure 3F).

No histopathological changes were observed in the liver tissue samples for any of the different treatments (data not shown).

**Lung biopersistence.** CNF material aggregates were clearly visible both free in the alveolar and bronchial spaces and inside alveolar macrophages at all studied timepoints (Figures 3B-E). The incidence of animals showing CNF and MWCNTs materials in the bronchia and alveolar space remained similar up to 90 days post-exposure (Tables S2-S4). The presence of CNF in the lung tissue at 90-d post-exposure was further confirmed by TEM analyses (Figure 4). Material similar to that identified as CNF was also present in the lung tissue at 24-h post-exposure, but not in samples from the vehicle group at 90-d post-exposure (data not shown).
The previously reported eosinophilic crystals were also observed inside macrophages of the BAL fluid (Figure 4C).

**Figure 4.** Transmission electron microscope (TEM) micrographs of mouse tissues 90 days after repeated pharyngeal aspiration with 14 μg/mouse/aspiration of U-CNF (A and C) or C-CNF (B). Presence of free CNF (black arrows) in the lung parenchyma (A and inset). Bronchoalveolar macrophage containing CNF (B and C, black arrows) and eosinophil crystals (C, red arrow).

**Genotoxicity.** Results on the induction of DNA damage, assessed by the comet assay, in BAL, lung and liver tissue by the different CNF samples at 28-d and 90-d post-exposure are shown in
Figure 5. Twenty-eight days after the exposure, only the lowest dose of U-CN (14 µg/mouse/aspiration) induced a statistically significant increase \((p = 0.031)\) in DNA damage in lung cells. At 90-d post-exposure, a significant increase in DNA damage was seen in BAL cells for all three doses of T-CN tested \((p < 0.001)\), with a significant linear dose-response \((p = 0.006, \text{ slope} = 0.030)\). On the other hand, the low, medium, and high doses of C-CN induced a significant increase in DNA damage in liver cells \((p < 0.05)\), with a statistically significant linear dose-response \((p = 0.0348, \text{ slope} = 0.023)\).

Figure 5. DNA damage (percentage of DNA in comet tail; mean ± SEM) in bronchoalveolar lavage (BAL), lung and liver cells of mice 28 and 90 days after repeated (3x) pharyngeal aspiration with the vehicle and the CNF samples. Asterisks designate statistically significant differences compared with the vehicle group at \(*p < 0.05, **p < 0.01, ***p < 0.001\) and \(****p < 0.0001\). The positive control, \(\text{H}_2\text{O}_2\) (20 mM), induced a statistically significant increase in the
percentage of DNA in tail over the negative control values in all the experiments performed (1.94 ± 0.4-fold increase; \( p < 0.001 \)) confirming the validity of the assay (data not shown).

The induction of DNA damage by the LPS-spiked samples was compared with that of the respective uncontaminated sample (T-CNФ at 14 µg/mouse/aspiration) in each tissue at both time points (Figure 6). Twenty-eight days after exposure, only the lowest LPS dose (0.02 ng/mouse/aspiration) induced a significant increase (\( p = 0.028 \)) in DNA damage in BAL cells. At 90-d post-exposure, a significant increase in DNA damage was induced in liver cells by all three doses of LPS (\( p < 0.05 \)), and the effect showed a significant linear dose-response (\( p = 0.034, \) slope = 0.017).

**Figure 6.** DNA damage (percentage of DNA in comet tail; mean ± SEM) in bronchoalveolar lavage (BAL), lung and liver cells of mice 28 and 90 days after repeated (3x) pharyngeal aspiration with T-CNФ (14 µg/mouse/aspiration) spiked with 0, 0.02, 1 and 50
ng/mouse/aspiration of lipopolysaccharide (LPS). Asterisks designate statistically significant
differences compared with the non-spiked sample at * \( p < 0.05 \), ** \( p < 0.01 \) and *** \( p < 0.001 \).
The positive control, \( \text{H}_2\text{O}_2 \) (20 mM), induced a statistically significant increase in the percentage
of DNA in tail over the negative control values in all the experiments performed (1.94 ± 0.4-fold
increase; \( p < 0.001 \)) confirming the validity of the assay (data not shown).

None of the tested CNF samples significantly increased the frequency of micronucleated
normochromatic erythrocytes (MNCEs) in peripheral blood at any of the post-administration
times (Table S5). The frequency of MNCEs reflects chromosome damage accumulated from the
beginning of the treatment until about 60 h before the blood sampling. In all treated groups, the
percentage of polychromatic erythrocytes (PCEs) among blood erythrocytes was similar to the
values of the vehicle groups, indicating that the CNFs did not show bone marrow toxicity. The
positive control treatment (MWCNTs and MMC) significantly increased the frequency of
MPCEs, by 12.7-fold in the 28-day series (\( p < 0.01 \)) and 11.6-fold in the 90-day series (\( p < 0.01 \))
in comparison with the vehicle groups. Due to the short exposure time (48 h before the blood
sampling), the effect of MMC in the micronucleus assay was analyzed from PCEs- which
assesses chromosome damage that occurred 36–48 h before the sampling- and the findings
confirmed the validity of the MMC treatment. MMC significantly reduced the percentage of
PCEs in the 28-day and 90-day series (\( p < 0.01 \) and \( p < 0.0001 \), respectively).

None of the LPS doses induced a significant increase in the frequency of MNCEs in peripheral
blood at any of the post-administration times, when compared with the corresponding zero dose
(T-CN at 14 µg/mouse/aspiration) (Table S5). For all LPS treatments, the percentage of PCEs
among blood erythrocytes was similar as in the corresponding zero dose groups, indicating that
the contamination with LPS did not induce bone marrow toxicity.
DISCUSSION

In this study, the pulmonary and systemic toxicity of three different types of CNFs (unmodified, carboxymethylated and TEMPO oxidized CNFs), produced from the same source of birch pulp fibers and processed in the same way, except for the surface modification steps, were assessed in repeated pharyngeal aspiration-exposed mice for up to 90 days after the last administration. In addition, we investigated the potential role of endotoxin contamination of the fibrils on the observed toxic effects.

The wide applicability of fibrillated cellulose is endowed by the introduced surface modifications. Material characterization indicated that the surface charges had a limited influence on the morphology of the fibers, given the mild condition of oxidation used. The fibrils were polydisperse in size (length and width). Fibril fragments were observed for all CNF types, as fibrillation is never fully efficient. The fibrillation yield ranged from 54% (U-CNf) to 78% (T-CNf), which indicates that the proportion of nanofibrils varied among the surface modified samples. Meanwhile, colloidal stability was observed, in agreement with the anionic nature of the samples (ζ-potential between -19 and -30 mV), which promotes electrostatic stabilization, as also reported in other studies. Our results also revealed that the nanofibrils produced by TEMPO-mediated oxidation, which is usually an effective method in individualizing CNFs, displayed a relatively smaller lateral dimension (width) compared to the other nanofibril types. However, the differences in size between the surface modified nanofibrils were not significant and the effects to be discussed below mainly relate to the proportion of micro- and nanofibrils and the type of surface chemical groups on them.
No remarkable alterations in the apparent crystallinity were observed for the fibrillated celluloses with different chemical modifications. A slight decrease in the degree of crystallinity of T-CNF was observed, which can partially be explained by the effect of the oxidation process.\textsuperscript{46} Crystallinity may scale with the stiffness of the fibrils, which is considered a key property of fibers’ pathogenicity.\textsuperscript{47} In fact, stiffness has been pointed out as one of the determinant features of some carbon nanotubes in mesothelioma induction.\textsuperscript{48} However, fibrillated cellulose is quite flexible, as compared with carbon nanotubes and cellulose nanocrystals and therefore may be more easily engulfed by macrophages and cleared from the body.\textsuperscript{49}

Unfortunately, most of the studies assessing the toxicity of fibrillated cellulose do not provide detailed characterization, especially regarding the fibrillation yield and the functional group density. Hence, the results cannot be easily compared. We approach our study based on the type of surface modification, acknowledging that fibrillated celluloses, even if produced using the same modification, do not necessarily track with each other given the differences in charges and other characteristics (surface groups density, fibrillation efficiency, etc.). This indeed highlights the complexity of cellulose nano- and microfibrils and the need for better standardization.

In the present study, the exposure to CNFs triggered a recruitment of inflammatory cells to the lungs. Mice exposed to CNFs and to MWCNTs (and MMC; positive control) displayed an increased influx of neutrophils into BAL one day after the last administration, indicating an acute inflammatory response. Moreover, CNFs - mainly U-CNF and T-CNF- triggered the recruitment of eosinophils into the airways. Supporting the finding in BAL, the presence of neutrophils and some eosinophils was detected in the lung tissue after all treatments, although the small sample size precluded finding statistically significant differences with the vehicle-treated group. We previously observed similar results with a T-CNF sample (carboxyl content of 1.07 mmol.g\textsuperscript{-1}),
administrated at similar doses as in the present study, which induced a neutrophilic influx in the small and large bronchia of mice 24-h after a single aspiration.\textsuperscript{20}

After 28 days, the initial CNF-triggered acute pulmonary reaction had notably attenuated, although a statistically significant influx of neutrophils into BAL was still observed for all CNFs as well as for the positive control. However, a significant recruitment of eosinophils into BAL was only detected with the middle dose of U-CNf and with MWCNTs. U-CNf also showed a significant number of neutrophilic aggregates and the highest prevalence of eosinophilia in the lung tissue. A similar behavior was previously described for inhaled bulk-sized cellulose fibers, which caused an initially high inflammatory response that subsided after 28-day recovery period.\textsuperscript{50} Our results also conform with those observed by Ilves \textit{et al.}\textsuperscript{15} and Hadrup \textit{et al.},\textsuperscript{22} who described a more modest immune reaction induced by CNFs at 28 days post-exposure as compared with the acute response observed after 24 h. However, opposite to these studies, carboxylation of CNF (caused by TEMPO oxidation) did not result in the present study in a lower inflammation in terms of neutrophilic influx in BAL as compared with the unmodified CNF.

To the best of our knowledge, no previous studies have assessed the pulmonary effects of CNF after 90 days post-exposure. Among the CNFs, only the highest dose of T-CNf induced a statistically significant influx of neutrophils into BAL at this time point. Interestingly, the total number of neutrophils was similar to that observed at 28 days post-exposure. On the other hand, the neutrophilic influx induced by the positive control dramatically dropped down from 28 to 90 days after exposure. Assuming that MWCNTs contained in the positive control were responsible of the pulmonary effects, at 90 days post-exposure the total number of neutrophils induced by 28 µg/mouse/aspiration of MWCNTs was similar to that induced by the highest dose of T-CNf (56
µg/mouse/aspiration), and significantly different from the values of the vehicle group. Although neither recruitment of eosinophils into BAL, nor incidence of eosinophilia in the mouse lungs were detected with any of the materials at this timepoint, macrophages containing eosinophil-derived crystals were observed with all CNF treatments. The presence of eosinophilic crystals without an inflammatory reaction was earlier reported by Ilves et al.\cite{15} at 28-d post-exposure of CNF. The crystals, which are similar to Charcot-Leyden crystals associated with chronic allergic asthma, are likely formed from the breakdown of earlier recruited eosinophils.\cite{41} The presence of eosinophils and eosinophil crystal suggests that CNF is able to induce a T-helper (Th) 2 type of inflammatory response, earlier seen in association with asbestos and high aspect ratio nanomaterials, but not with granular nanomaterials.\cite{41,51,52} Conversely, a Th1-like immune response was described in BALB/c mice exposed to CNF, whereas a Th2-type immunity was observed after exposing the animals to asbestos.\cite{21}

The presence of endotoxins in CNF has been suggested as a possible reason for the inflammatory response observed in mice.\cite{14,15} However, endotoxin contamination did not explain the inflammatory effects observed in the present study, as the inflammatory potential of the LPS-spiked T-CNFi samples did not differ from the corresponding uncontaminated T-CNFi sample, and no LPS dose dependency was seen. The tested LPS dose range (0.02 to 50 ng/mouse/aspiration) corresponds to 4 to 10^4 EU mL^{-1}, which clearly exceeds the reported levels of nanomaterials’ contamination.\cite{26}

In the present study, CNF-induced local genotoxic effects were assessed by measuring DNA damage in BAL and lung cells. In addition, systemic genotoxicity was assessed by measuring DNA damage in liver cells and the induction of micronuclei in peripheral blood erythrocytes. At 28 days after the exposure, we only detected a significant increase in DNA damage for the
lowest dose of U-CN (14 µg/mouse/aspiration) in lung cells, without a significant linear dose-response. In addition, the lowest dose of LPS-spiked T-CN sample (0.02 ng LPS/mouse/aspiration) increased DNA damage in BAL cells, without a linear dose-response. At 90 days after the exposure, a significant increase in DNA damage was induced by all doses of T-CN and C-CN, in BAL and liver cells, respectively. A significant increasing linear dose-response was observed with both materials. In addition, the three doses of LPS-spiked T-CN induced DNA damage in liver cells, showing a significant positive dose-response. Interestingly, neither T-CN nor C-CN induced DNA damage at 28 days post-exposure. As the comet assay depicts the level of DNA damage at the time of sampling without showing cumulative effects, the rate of DNA damage or the ratio of DNA damage and DNA repair is higher after 3 months than 1 month for T-CN in BAL cells and for C-CN in the liver. Although we do not have information on time points between 28 and 90 days or later, this may indicate that the longer these CNFs stay in the body, the higher the level of continuous DNA damage will be.

Carboxylation and carboxymethylation of CNF have been associated with lower pulmonary DNA damage in mice intratracheally or oropharyngeal treated with modified CNFs compared with mice treated with enzymatically pre-treated CNFs. However, none of the materials that were evaluated in vivo induced genetic damage in human bronchial epithelial BEAS-2B cells, suggesting that the mechanisms involved in the genotoxic effects detected in vivo were not present in the in vitro model. Similarly, none of the CNF samples evaluated by Aimonen et al. - including a non-modified enzymatically pre-treated CNF, as well as one CNF sample of the same surface chemistry as C-CN increased the frequency of DNA damage or micronuclei in the same cell model. Conversely, the same authors have recently reported a significant induction of micronuclei after treating BEAS-2B cells with the coarse fraction of a
carboxymethylated fibrillated cellulose produced in the same way than in the present study. However, neither the medium and fine fractions of the carboxymethylated CNF, nor any of the size fractions of an unmodified and a TEMPO oxidized CNF, also produced in a similar way that the ones evaluated in this study, induced any genotoxic effect. These findings suggest that the surface chemistry and the size of the fibrils may modulate the capacity of CNF to induce genotoxic effects by primary mechanisms. Primary genotoxicity is due to an interaction of the material with the target cells, either by directly damaging the DNA molecule or related proteins, or indirectly by interacting with other cellular organelles (e.g., generating oxidative stress). However, genotoxicity may also raise by secondary mechanisms, mediated by inflammation or other intermediate responses not present in the \textit{in vitro} models. The \textit{in vivo} genotoxicity may also be due to processes that require longer-term assessment than \textit{in vitro} assays allow. For instance, a depletion of the anti-oxidant defenses or DNA repair systems. In fact, Ventura \textit{et al.} reported that a TEMPO oxidized CNF increased the frequency of micronuclei in adenocarcinomic human alveolar epithelial A549 cells co-cultured with acute monocytic leukemia THP-1 macrophages, a cellular system that has been reported to allow the detection of some mechanisms of secondary genotoxicity. However, as both cell types in this co-culture system were simultaneously treated with CNF, the involvement of primary genotoxic mechanisms could not be rule out. On the other hand, our finding that T-CNF was the only cellulosic material that still induced an inflammatory response in BAL cells after 90 days supports the idea of a secondary induction of DNA damage - mediated by inflammation – for this material.

The fact that both C-CNF and LPS-spiked T-CNF were able to induce DNA damage in liver at 90 days after exposure may indicate that liver cells might be more sensitive than lung cells to either primary or secondary mechanisms of genotoxicity. To show primary genotoxicity, CNF
should first be translocated to the liver. Identification of CNF in biological samples is technically challenging.\textsuperscript{4,32} CNF would need to be tagged with e.g., fluorescent tags\textsuperscript{55} that can affect their surface properties.\textsuperscript{56} Hence, we could not detect the materials in the liver. However, other fibrous nanomaterials, \textit{e.g.}, MWCNTs, have been reported to be translocated to the liver and induce DNA damage that could still be detected one year after a single intratracheal instillation.\textsuperscript{57} Hepatic genotoxic effects could be caused by the ability of the translocated materials to induce oxidative stress,\textsuperscript{58} which has been reported to be the main deleterious effect of nanomaterials.\textsuperscript{59,60} Alternatively, the genotoxic effects observed in the liver could be caused by circulating inflammatory mediators released during pulmonary inflammation.\textsuperscript{58} The former hypothesis would be supported by the fact that we observed hepatic DNA damage only at the longest time point, which may reflect the time needed for the materials to translocate in sufficient amounts from lungs to the liver.\textsuperscript{58} In fact, neither the carboxylated nor the enzymatically pre-treated CNFs evaluated by Hadrup \textit{et al.}\textsuperscript{22} induced hepatic DNA damage 28 days the intratracheal instillation, and the authors assumed that this was because the materials had not reached the liver. On the other hand, the carboxymethylated CNF assessed by Aimonen \textit{et al.}\textsuperscript{53} showed the most effective induction of radical oxygen species (ROS) in BEAS-2B cells, compared with the other surface modified CNFs, in their respective size fractions. Another carboxymethylated CNF (same surface chemistry as C-CNf) was also able to induce the formation of ROS in a dose-dependent manner in BEAS-2B cells,\textsuperscript{19} although not in THP-1 macrophages.\textsuperscript{3} Similarly, two different CNFs neither induced ROS generation or cytotoxic effects in Kupffer cells,\textsuperscript{55} where particles deposited in the liver are primary accumulated.\textsuperscript{61} However, no surface modification was reported for any of these CNFs. Furthermore, LPS could have triggered the formation of ROS in liver cells if it were carried by translocated T-CNf, although T-CNf itself would not have induce ROS formation. In
fact, endotoxins activate Kupffer cells triggering a cascade of biochemical signals in these cells that result in cytokine and ROS production.\textsuperscript{62} Regarding the second hypothesis, the original pulmonary inflammation triggered by LPS-spiked T-CNF samples was no longer relevant after 1 month, whereas C-CNF-induced pulmonary inflammation showed similar values than the vehicle group. Hence, an inflammation-mediated mechanism does not seem to be involved in these cases.

It is worth noticing that T-CNF and C-CNF did not show big differences in the proportion of nanofibrils (measured by the fibrillation yield), the nanofibrils’ lateral size, the surface charge and the functional group densities. However, these small differences, or differences in other parameters related to the surface modification process, seem to influence the biological behavior of the materials. Nevertheless, whatever the mechanisms of action involved, the observed increases in DNA damage were not pronounced, although the effects were dose-dependent in all the cases. The fact that these effects were only detected at the latest timepoint may also reflect a depletion of the defense mechanisms (e.g., anti-oxidant mechanisms or DNA repair systems) with time as a consequence of continuous DNA damage. If DNA damage persists, it may lead in the long-term to carcinogenicity.

All the CNF samples, as well as MWCNTs, showed a high biopersistence in the lung tissue. This is in agreement with previous studies with cellulose-based materials.\textsuperscript{50} No clearance or degradation of the CNFs present in the mouse lungs was reported to occur within 1 month after exposure.\textsuperscript{15} Although CNFs were not quantified in the present study, we observed a similar incidence of animals showing the CNFs in the bronchioles and alveolar spaces within the 90-d period. C-CNF had the lowest incidence, in agreement with the previously reported lower number of agglomerates and smaller total area of agglomerates of this CNF in the lung compared
with the other CNF types studied by Ilves et al.\textsuperscript{15} As the initial administrated dose was the same for all the materials, these findings could reflect a lower agglomeration status of C-CNF, individualized nanofibrils being more difficult to be detected. However, opposite to MWCNTs – which also showed high biopersistence- no granuloma formation was observed in any of the CNF-treated mice. Similar results were previously reported for the CNFs analyzed by Ilves et al.\textsuperscript{15} Therefore, although CNF showed some features of a Th2 type of inflammatory response that is also associated to other high aspect ratio nanomaterials, our findings support the hypothesis that the toxic pulmonary response induced by CNF may differ from that caused by carbon nanotubes or asbestos.\textsuperscript{12, 21} The difference may partly be due to the higher flexibility of CNF, which will not result in frustrated phagocytosis unlike stiff fibers (\textit{e.g.}, asbestos).\textsuperscript{21}

\textbf{CONCLUSIONS}

In summary, the findings presented here suggest that different parameters related to the surface modification of CNF (surface functional group density, fibrillation yield,\ldots) can affect the evolution of the acute inflammatory response and the generation of DNA damage induced by the material when administrated to the lungs. Furthermore, CNF might be translocated from the lungs to the liver causing DNA damage. The high biopersistence of CNF, together with the reported genotoxic effects, raise concerns about potential carcinogenicity in a long-term pulmonary exposure. The carcinogenicity might affect not only the lungs, as the portal of entry, but also other organs and tissue, \textit{e.g.}, liver, where the nanofibers could be translocated. Therefore, further long-term studies are needed to elucidate the effects and modes of action of different types of CNF. These studies should be combined with exposure measurements that could allow performing an appropriate assessment of the associated risks.
ASSOCIATED CONTENT

Supporting Information

Field emission scanning electron microscopy images of CNFs (Figure S1); body weight gain in mice exposed to CNF samples (Figure S2); bronchoalveolar lavage fluid cellularity after CNF exposure (Table S1); histological analyses of lung samples at 1-, 28- and 90-day post-exposure to CNFs (Tables S2, S3 and S4); frequency of micronucleated peripheral blood erythrocytes after CNF exposure (Table S5).

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Effect of Surface Modification on the Pulmonary and Systemic Toxicity of Cellulose Nanofibrils

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