Työterveyslaitos | Arbetshälsoinstitutet
Finnish Institute of Occupational Health

TIETOA TYÖSTÄ

Hazard characterization of graphene-based nanomaterials in energy production and storage (GrapHazard)

FINAL REPORT OF THE RESEARCH PROJECT

Marco Pelin
Adriana Rodríguez-Garraus
Clara Passerino
Mari Venäläinen
Michela Carlin
Gerard Vales
Silvio Sosa
Satu Suhonen
Cristina Ponti
Kukka Aimonen
Josefa Domenech
Aurelia Tubaro
Julia Catalán

Hazard characterization of graphene-based nanomaterials in energy production and storage (GrapHazard)

FINAL REPORT

Marco Pelin[1], Adriana Rodríguez-Garraus[2], Clara Passerino[1], Mari Venäläinen[2], Michela Carlin[1], Gerard Vales[2], Silvio Sosa[1], Satu Suhonen[2], Cristina Ponti[1], Kukka Aimonen[2], Josefa Domenech[2], Aurelia Tubaro[1], and Julia Catalán[2]

[1]Department of Life Sciences, University of Trieste, Trieste, Italy

[2]Finnish Institute of Occupational Health, Helsinki, Finland

Finnish Institute of Occupational Health

Occupational Safety

PB 40

00251 Helsinki

www.ttl.fi

Photos: Tomi Kanerva (Figure 11)

This project has been accomplished with the support by the Finnish Work Environment Fund (grant no. 200338), the Italian National Institute for Insurance against Accidents at Work (INAIL), the Finnish Institute of Occupational Health, and the University of Trieste in the frame of the SAF€RA 2020 program (GrapHazard).

ISBN 978-952-391-136-9 (PDF)

ABSTRACT

Graphene-based materials (GBMs) are two-dimensional carbon-based compounds that have raised an increasing interest over the last years due to its unique physicochemical (PC) properties, such as hardness, flexibility, and high thermally and electrically conductivity, making them attractive for a multitude of different industrial applications, especially in the energy storage sector. However, the same PC properties that confer GBMs extraordinary functionalities also affect their toxic response. The main risk for human health appears to be associated with occupational exposure to GBMs through inhalation during their production, use, and waste disposal.

The aim of the present project was to identify and characterize the hazard posed by GBMs used in energy production and storage by i) assessing the cytotoxicity of GBMs towards human bronchial cells using adapted toxicity testing assays, ii) elucidating the involved mechanisms of action, and iii) investigating how the PC properties of GBMs can modulate the toxic response. For that, two different case-studies were implemented. The first one evaluated the effect of chemical reduction and formulation on the toxicity of graphene oxide (GO). In the second case-study, reduced graphene oxides (rGOs) with different oxidation status, number of layers and lateral size were toxicologically assessed.

Our findings suggest that the cytotoxic and genotoxic potential of GBMs towards bronchio-epithelial cells depends on the production process and the PC properties of the materials. The reduction process applied to GO seems to induce a reduction of cell viability and an increase in the generation of ROS when the cells are exposed to these materials. On the other hand, a well-dispersed and stable water-based formulation of GO appears to prevent the material to be internalized into the cells and exert the cytotoxic effects observed for the same powdered GO. On the other hand, the results with different rGOs, indicate that although it is not possible to establish a direct correlation between the oxidation status of the materials and any of the hazard endpoints that were assessed, there seems to be an association bewteen higher oxygen content and higher cytotoxicity as well as early ROS induction. On the contrary, genotoxic effects were observed for the rGO of lowest density of oxygen groups. On the other hand, the cytotoxicity and ROS production potentials may be decreased by increasing the number of layers of the rGOs, whereas the lateral size does not seem to have an influence. The observed results can be of interest when considering the safe-by-design production of new GBMs. Finally, due to the limited available information on airborne GBM concentrations and human biomonitoring, minimization of exposure to GBMs by applying the most appropriate occupational hygiene measures is highly recommended.

TIIVISTELMÄ

Grafeenipohjaiset materiaalit (GPM) ovat kaksiulotteisia hiilipohjaisia yhdisteitä, jotka ovat herättäneet kasvavaa kiinnostusta viime vuosina niiden ainutlaatuisten fysikaalis-kemiallisten (FK) ominaisuuksien, kuten kovuuden, joustavuuden ja korkean lämmön- ja sähkönjohtavuuden, vuoksi, mikä tekee niistä houkuttelevia erilaisten teollisten sovellusten näkökulmasta, erityisesti energian varastoinnin saralla. Samat FK-ominaisuudet, jotka mahdollistavat GPM:ien poikkeukselliset toiminnallisuudet, vaikuttavat kuitenkin myös niiden toksiseen vasteeseen. Suurin riski ihmisten terveydelle vaikuttaa liittyvän työperäiseen altistumiseen hengitysteitse GPM:ien tuotannon, käytön ja jätteiden hävittämisen aikana.

Tämän hankkeen tavoitteena oli tunnistaa ja karakterisoida energian tuotannossa ja varastoinnissa käytettävien GPM:ien aiheuttama vaara i) arvioimalla GPM:ien solumyrkyllisyyttä ihmisen keuhkoputkien soluja kohtaan sovellettuja toksisuustestausmenetelmiä käyttäen, ii) selvittämällä siihen liittyvät vaikutusmekanismit ja iii) tutkimalla, kuinka GPM:ien FK-ominaisuudet voivat vaikuttaa niiden toksiseen vasteeseen. Tätä varten toteutettiin kaksi tapaustutkimusta. Ensimmäisessä arvioitiin kemiallisen pelkistyksen ja formuloinnin vaikutusta grafeenioksidin (GO) toksisuuteen. Toisessa tapaustutkimuksessa arvioitiin toksikologisesti pelkistettyjä grafeenioksideja (rGO), joiden hapetusluku, kerrosten lukumäärä ja lateraalinen koko vaihtelivat.

Tuloksemme viittaavat siihen, että GPM:ien solumyrkyllinen ja genotoksinen potentiaali keuhkoputkien epiteelisoluja kohtaan riippuu niiden tuotantoprosessista ja materiaalin FK-ominaisuuksista. GO:n pelkistysprosessi näyttää aiheuttavat solujen elinkyvyn heikentymistä ja lisäävän ROS:n muodostumista, kun solut altistetaan näille materiaaleille. Toisaalta hyvin dispergoitunut ja stabiili GO:n vesipohjainen formulaatio vaikuttaa estävän materiaalin sisäänoton soluihin ja solumyrkyllisten vaikutuksien aikaansaannin, mitä havaitaan vastaavan jauhemaisen GO:n kanssa. Kuitenkin tulokset erilaisilla rGO:illa osoittavat, että vaikkei ole mahdollista osoittaa suoraa korrelaatiota materiaalien hapetusluvun ja mitattujen toksisten vasteiden välillä, materiaalin korkeamman happipitoisuuden ja kohonneen solumyrkyllisyyden sekä varhaisen ROS-induktion välillä näyttää olevan yhteys. Sitä vastoin happitiheydeltään pienimmällä rGO:lla havaittiin olevan genotoksisia vaikutuksia. Toisaalta rGO:iden kerrosten lukumäärän lisääntyminen saattaa vähentää materiaalien solumyrkyllisyyttä ja ROS-tuotantopotentiaalia, kun taas lateraalisella koolla ei näytä olevan vaikutusta. Saadut tulokset voivat olla kiinnostavia, kun pohditaan uusien GPM:ien tuotantoa safe-by-design -periaatteen mukaan. Lopuksi, koska aineistoa ilman GPM-pitoisuuksista ja ihmisen biomonitoroinnista on saatavilla rajoitetusti, on erittäin suositeltavaa minimoida altistuminen GPM:ille käyttämällä sopivimpia työhygieniatoimenpiteitä.

RIASSUNTO

I materiali a base di grafene (GBMs) sono composti bidimensionali a base de carbonio che negli ultimi anni ha suscitato un crescente interesse grazie alle sue uniche proprietà fisico-chimiche, come durezza, flessibilità ed elevata conduttività termica ed elettrica, che li rendono attraenti per una moltitudine di diverse applicazioni industriali, in particolare nel settore dello stoccaggio dell'energia. Tuttavia, le stesse proprietà che conferiscono ai GBMs funzionalità straordinarie influenzano anche la loro risposta tossica. Il rischio principale per la salute umana sembra essere associato all'esposizione professionale ai GBMs attraverso l'inalazione durante la loro produzione, utilizzo e smaltimento dei rifiuti.

Lo scopo del presente progetto era identificare e caratterizzare il pericolo rappresentato dai GBMs utilizzati nella produzione e nello stoccaggio di energia i) valutando la citotossicità dei GBM nei confronti delle cellule bronchiali umane utilizzando test di tossicità adattati, ii) chiarendo i meccanismi d'azione coinvolti e iii) studiando come le proprietà fisico-chimiche dei GBMs possano modulare la risposta tossica. A tal fine sono stati implementati due diversi casi studio. Il primo ha valutato l'effetto della riduzione e della formulazione chimica sulla tossicità dell'ossido di grafene (GO). Nel secondo caso di studio, sono stati valutati ossidi di grafene ridotti (rGO) con diverso stato di ossidazione, numero di strati e dimensione laterale.

I nostri risultati suggeriscono che il potenziale citotossico e genotossico dei GBMs nei confronti delle cellule bronco-epiteliali dipende dal processo di produzione e dalle proprietà fisico-chimiche dei materiali. Il processo di riduzione applicato al GO sembra indurre una riduzione della vitalità cellulare e un aumento della generazione di ROS quando le cellule sono esposte a questi materiali. D'altro canto, una formulazione di GO a base acquosa ben dispersa e stabile sembra impedire al materiale di essere internalizzato nelle cellule ed esercitare gli effetti citotossici osservati per lo stesso GO in polvere. D'altra parte, i risultati con diversi rGO indicano che, sebbene non sia possibile stabilire una correlazione diretta tra lo stato di ossidazione dei materiali e uno qualsiasi degli endpoint valutati, sembra esserci un'associazione tra un contenuto di ossigeno più elevato e maggiore citotossicità e induzione precoce di ROS. Al contrario, sono stati osservati effetti genotossici per l'rGO con la densità più bassa di gruppi funzionali contententi ossigeno. D'altra parte, la citotossicità e il potenziale di produzione di ROS possono essere diminuiti aumentando il numero di strati delle rGO, mentre la dimensione laterale non sembra avere una significativa influenza. I risultati osservati risultano interessanti se si considera una sicura produzione di GBMs fin dalla loro progettazione. Infine, a causa delle limitate informazioni disponibili sulle concentrazioni di GBMs nell'aria e sul biomonitoraggio umano, è altamente raccomandato ridurre al minimo l'esposizione ai GBMs applicando le misure di igiene professionale più appropriate.

TABLE OF CONTENTS

1 BACKGROUND

1.1 Graphene and its use in energy applications

Graphene is a two-dimensional material consisting of a monolayer of carbon atoms arranged in a honeycomb-like structure, with a high surface area on both sides of the planar axis (Geim & Novoselov, 2007; M. Pelin et al., 2018). Since its isolation in 2004 by mechanical exfoliation of graphite, the interest in graphene has been progressively increasing over the years due to its unique physicochemical properties. Being 100 times stronger than steel, graphene is yet enormously flexible, extremely thermally and electrically conductive, and impermeable to all gases (Alwarappan et al., 2009; Balandin et al., 2008; Lee et al., 2008; Park et al., 2017). Oxidation and/or functionalization of graphene can generate a wide family of graphene-based materials (GBMs), endowed with very different physico-chemical (PC) properties that make them compatible and attractive for a multitude of different applications. In fact, GBMs are one of the most promising tools in the development of batteries, supercapacitors, and solar cells (Brownson et al., 2011; El-Kady et al., 2016; Tarelho et al., 2018). GBMs are also applied in advanced food packaging, foldable touch screens and superprotective coatings for wind turbines and ships (Park et al. 2017), as well as in biomedical applications, such as drug delivery systems, biosensors, anti-bacterial agents, tissue engineering, and imaging systems (Guo et al., 2021; Magne et al., 2022).

The graphene market is probably one of the most active among all nanomaterials' markets. Thanks to the huge number of resources invested by the European Commission on achieving graphene commercialization though the EU Graphene Flagship program[1], the European market is currently the biggest at the global level, although other markets, especially China, are catching up (EUON, 2022a). In 2020, the EU graphene market was reported to have a size of around 0.04 Kilotons per volume, with a value of 92.8 €million[2]. Furthermore, the size of the global graphene market is estimated to grow at the annual rate of almost 40 percent from 2020 to 2027.

Due to its exceptional properties, graphene has multiple potential applications in the energy sector, especially in relation to energy storage, as it can increase the performance, functionality, and durability of current energy storage devices (Figure 1). The four main energy-related areas where graphene will have an important impact in the future are: solar cells, supercapacitors, lithium-ion batteries, and catalysis for fuel cells (Luo et al.,

[1] https://graphene-flagship.eu/

[2] Europe Nanomaterials Market Trends, Analysis, Growth, Size and Share (inkwoodresearch.com)

2012; Olabi et al., 2021; Xiang et al., 2021). For instance, graphene could dramatically increase the lifespan of a traditional lithium-ion battery, meaning that devices can be charged quicker and hold more power for longer. Another example is the use of graphene supercapacitors, which could reduce the weight of cars or planes, as they are lighter than the current ones, while they could provide much more power using less energy than conventional devices. An overview of the potential applications of GBMs in this field is giving in the video prepared by Manchester University, where graphene was discovered[3].

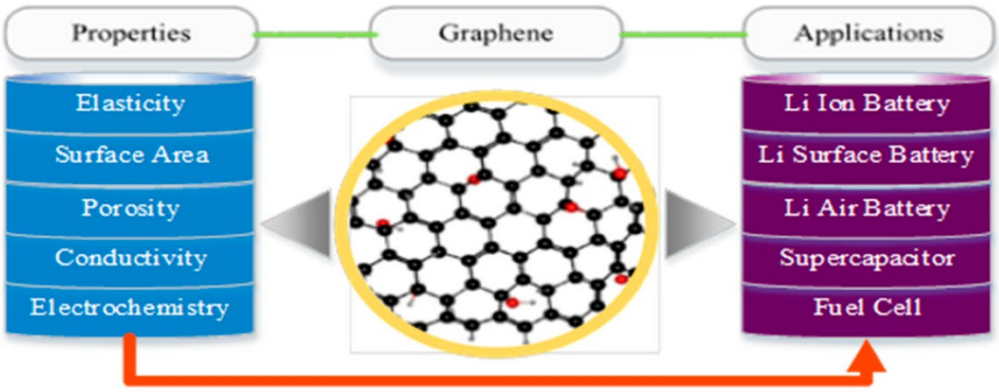


Figure 1. Energy-related applications of graphene with unique properties. Reprinted from (Xiang et al., 2021). Copyright 2021 by the authors.

1.2 The graphene-based materials family

Graphene and its related materials constitute a broad family, the graphene-based materials (GBMs), with very different PC properties. Therefore, the European Union Graphene Flagship project suggested a classification framework based on three main PC descriptors: the number of graphene layers, the average lateral size, and the carbon-to-oxygen (C/O) ratio (Wick et al., 2014). Based on these parameters, different authors have proposed classifying GBMs into graphene oxide (GO), reduced graphene oxide (rGO), few-layer graphene (FLG), graphene nanosheets and flakes, and graphene ribbons and dots (Domenech et al., 2022). Some of these materials are shown in Figure 2. In addition, the planar surface of graphene allows functionalization with, e.g., carbonyl, hydroxyl, and

[3] Energy - Graphene - The University of Manchester

epoxy groups, or with capping agents or coatings, such as polyethylene glycol, to make it more compatible with its applications (Park et al., 2017).

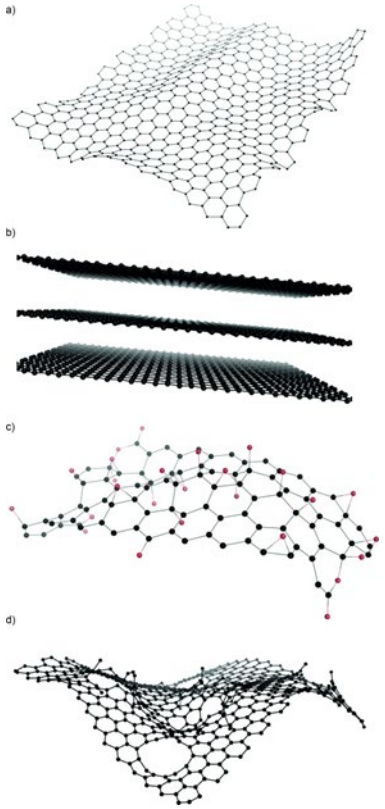


Figure 2. Representative chemical structures of some of the graphene-based materials: (a) graphene, (b) few-layer graphene, (c) graphene oxide (oxygen atoms are in red) and (d) reduced graphene oxide. Reprinted with permission from (Bianco, 2013). Copyright 2013 Wiley Online Library.

Among the broad variety of GBMs, GO stands out as the most widely used and biologically relevant material because of its good dispersibility in organic solvents and matrices, as well as its efficient functionalization (Achawi et al., 2021; Ray, 2015; Reina et al., 2017). On the other hand, rGO can be obtained from GO by removal of some oxygen-bearing functional groups using reducing chemical agents or thermal reduction methods (Huang et al., 2011; Pei & Cheng, 2012; Razaq et al., 2022). The number of the remaining oxygen-containing functional groups can be controlled, which allows

modulating the dispersibility and electrical performance of rGO, making it suitable for different applications (Ray, 2015; Razaq et al., 2022).

1.3 Physico-chemical and biological properties of graphene-based materials

As described in the previous section, the different members of the large family of GBMs are endowed with different PC characteristics. However, the same PC properties that confer GBMs extraordinary functionalities also guide their interaction with biological systems and may affect the potential toxic response of these compounds (Magne et al., 2022).

There is already an extensive literature on the health effects of GBMs, e.g., see reviews by (Domenech et al., 2022; Fadeel et al., 2018; M. Pelin et al., 2018) or the recent report from the European Observatory for Nanomaterials (EUON, 2022b) . All these studies reveal that the main risk to human health appears to be associated with occupational exposure to GBMs through inhalation during their production, use, and waste disposal. Pulmonary inflammation, fibrosis, and long bio-persistence in rodents have been observed in some *in vivo* inhalation toxicity studies (Fadeel et al., 2018; Lee et al., 2019; M. Pelin et al., 2018). Tentatively, some GBMs might have similar toxic properties to carbon nanotubes, some of which are known to be genotoxic and carcinogenic (Domenech et al., 2022). Furthermore, the studies also show evidence that human toxic effects of GBMs depend on their PC characteristics (EUON, 2022b). Among the most studied PC properties, degree of oxidation, thickness, agglomeration, and size play a relevant role on toxicity, although in the latter case the available information is contradictory regarding the observed effects (Achawi et al., 2021). For instance, the cytotoxic response of GO in different cell systems has shown to be dependent on the flake size (Gies et al., 2019) and the number of layers (Yang et al., 2020). As concerns rGO toxicity, the reduction method used, lateral size and oxygen-containing functional groups have been reported to affect the *in vitro* outcomes (Akhavan et al., 2012; Mittal et al., 2016; Ou et al., 2021). However, the broad variability of materials, which are often poorly characterized (Achawi et al., 2021; M. Pelin et al., 2018), and of cellular systems used, preclude a clear identification of PC parameters that could drive the toxic response of GBMs (Bianco et al., 2013; Domenech et al., 2022).

2 AIMS OF THE STUDY

The main aim of this project was **to identify and characterize the hazard posed by GBMs used in energy production and storage**. This aim was achieved through the following objectives:

1. *Adapt toxicity test guidelines (TGs) for advanced materials and apply them for GBMs testing*
   By employing the latest principles and procedures developed within the OECD Manufactured Nanomaterials Working Party (MNMWP) program and comparing *in vitro* results with the human biomonitoring data on the same toxicological endpoints in collaboration within the EU Graphene Flagship project.

2. *Contribute to elucidate the mechanisms of action at the basis of human toxic responses after inhalation exposure to GBMs*
   By using *in vitro* approaches able to differentiate between primary (interaction with target cellular components) and secondary (mediated by an inflammatory response) mechanisms of actions.

3. *Assess how the physico-chemical (PC) properties of GBMs can affect their toxicity*
   By evaluating GBMs with different PC properties for their *in vitro* effects on targeted cells, providing data that can be used in selecting safer materials in energy production and storage applications (Safe-by-Design approaches).

The work in the project was organized in seven different tasks, which are described in detail in Annex I. Furthermore, the described objectives were implemented by setting up two different case-studies. The first one evaluated the effect of chemical reduction and formulation on the toxicity of GO. In the second case-study, rGOs with different oxidation status, number of layers and lateral size were toxicologically assessed.

3 MATERIALS AND METHODS

3.1 Graphene-based materials

A panel of 9 different GBMs, comprising mainly GO and rGO, were tested to (i) characterize the hazard posed by GBMs at the pulmonary level and (ii) characterize the role of the different PCl properties on GBMs' toxicological potential.

In the case-study 1, three materials were initially considered to investigate the role of two important features of GO, potentially affecting its safety profile: (i) its chemical reduction and (ii) its different formulation as powder or in a stable water dispersion form. In particular, commercially available powder GO, prepared through a modified Hammers' method, its chemically reduced form obtained using ascorbic acid (rGO) and its stable water dispersion form (wdGO) prepared by subjecting GO to a dilution and to an ultrasound process were provided by one manufacturer and tested in GrapHazard.

In the case-study 2, six different rGO in powder form were prepared by thermochemical reduction of GO and provided by another company. These materials were studied to investigate the role of three key PC properties on the *in vitro* effects of rGO: (i) C/O ratio, as an index of the amount of O_2-bearing functional groups on rGO structure; (ii) lateral size and (iii) number of layers.

Each material was dispersed in 0.1% bovine serum album (BSA) solution to achieve dispersions to be further diluted directly in cell media, allowing cells treatment.

3.2 Characterization of the materials

Each material was fully physico-chemically characterized by different techniques. Elemental analysis was performed to evaluate the atomic composition of each material and allowed the calculation of their C/O ratio. The C/O ratio was also calculated using X-ray diffraction (XRD). The presence of O_2-bearing functional groups on material structures was evaluated also by thermogravimetric analysis (TGA), while the graphene structure was determined by Raman spectroscopy. Depending on the studied material, lateral dimension was evaluated by Laser diffraction & Dynamic Light Scattering (DLS) and/or transmission electron microscopy (TEM). The latter was used also to determine the shape of each flake, evaluated also by light microscopy. The number of graphene layers in a stack was determined through specific surface area (SSA) measured by the Brunauer-Emmett-Teller technique (BET).

Endotoxin contamination of each material was assessed by a modified version of the Tumor Necrosis Factor (TNF)-α Expression Test (TET) assay using macrophages obtained

by differentiation of human THP-1 monocytes (Pelin et al., 2023). The amount of endotoxin was calculated on the basis of TNF-α cell release induced by LPS content in each material.

Once dispersed in 0.1 % BSA, each material was analyzed for the dispersion stability by UV-Vis analysis up to 2 h. The analysis of pH of each dispersion excluded any bias due to acidic behavior. Table 1 shows the main PC properties of each material used in both case-studies.

Table 1. Characteristics of the graphene-based materials tested in GrapHazard

Material	C/O ratio	Lateral dimension (nm)			No. of layers	Dispersion stability (2h)	Endotoxin content#
		TEM (average)	TEM (range)	DLS			
Materials of case-study 1							
GO	1.2*	2476	124 - 8792	-	-	Yes	No
rGO	5.3*	3231	376 - 8552	-	-	Yes	No
wdGO	1.2*	1005	127 - 3256	-	-	Yes	No
Materials of case-study 2							
rGO1	52.6**	2387	280 - 10499	39000	4	Yes	No
rGO2	13.9**	2083	165 - 9089	40000	4	Yes	No
rGO3	11.0**	1191	169 - 3573	39000	4	Yes	No
rGO4	7.1**	1927	274 - 8140	42000	4	Yes	No
rGO5	45.5**	2368	380 - 13655	< 1000	5	Yes	No
rGO6	66.7**	4282	449 - 16659	43000	8	No (up to 1 h)	No

* C/O ratio computed using elemental analysis data
** C/O ratio computed using XRD data
# No: endotoxin content < 0.5 EU/mL (acceptable limit suggested by the U.S. FDA for medical devices (FDA, 2012)
DLS: Dynamic Light Scattering; TEM: Transmission Electron Microscopy; XRD: X-ray diffraction

3.3 Toxicity assessment: primary effects

3.3.1 Cell line

Toxicity assessment of primary effects was carried out on human epithelial bronchial cells. To this aim, the 16HBE14o− cell line was kindly provided by Dr. Dieter Gruenert's lab (University of California; San Francisco, CA, USA) and cells were cultured in standard conditions in a humidified incubator at 37 °C with 5% CO_2. This cell line was originally obtained from a 1-year-old male and immortalized with SV40 plasmid. It is widely used as a model for respiratory epithelial diseases and barrier function since it retains many of the functions and morphology of differentiated normal bronchial epithelial cells (Callaghan et al., 2020).

3.3.2 Cellular uptake

The aim of these experiments was to confirm uptake of the graphene derivatives by bronchial cells as such internalization is requested for a proper interpretation of the toxicity results, e.g., to ensure that a negative genotoxic outcome is not due to a lack of interaction of the material with the cellular genetic material or organelles if the material is not uptaken by the cells (Doak et al., 2023).

Cellular internalization of GBMs was evaluated using fluorescence confocal microscopy exploiting the light reflection properties of the materials to detect them into cells, as previously reported (Chortarea et al., 2022; Pelin et al., 2017). However, quantitative differences in the level of internalization among the different rGOs could not be assessed due to the limitations of the method.

3.3.3 Toxicological methods adjusted to graphene-based materials

GBM toxic potential on 16HBE14o− bronchial cells was investigated after a short (3 h) and a longer (24 h) exposure time evaluating different toxicological endpoints (Figure 3). In particular:

1. Cell viability was evaluated by the WST-8 reduction assay, a colorimetric test able to measure viable cells by means of mitochondrial activity. This test was chosen because previous studies demonstrated that it does not give any interferences with GBMs (Liao et al., 2011).
   Cell viability was also assessed using the CellTiter-GloVR luminescent assay as this method has previously used with nanomaterials (Aimonen et al., 2021) as a preliminary test to set the range of concentrations that should be included in the genotoxicity assays, according to the OECD test guidelines (OECD, 2023).

2. The generation of reactive oxygen species (ROS) was evaluated using the fluorescence DCFDA probe, already used in previous studies with a protocol minimizing interferences with GBMs (Marco Pelin et al., 2018).
3. Pro-inflammatory effect was investigated by measuring the cellular release of 12 pro-inflammatory mediators among those mostly relevant in a pulmonary inflammation, including cytokines and chemokines, using commercially available enzyme-linked immunosorbent (ELISA) assays.
4. Genotoxic effects, evaluating DNA and chromosome damage, were assessed by the alkaline comet assay and the cytokinesis-block micronucleus test, respectively. The former has been extensively used for testing nanomaterials and improved for avoiding interferences' problems (El Yamani et al., 2022). The latter is a validated method (OECD TG487) that has recently been adapted for testing nanomaterials (OECD, 2022). Within GrapHazard, a flow cytometry-based version of the micronucleus assay, which includes a thorough assessment of potential interference sources, was set up.

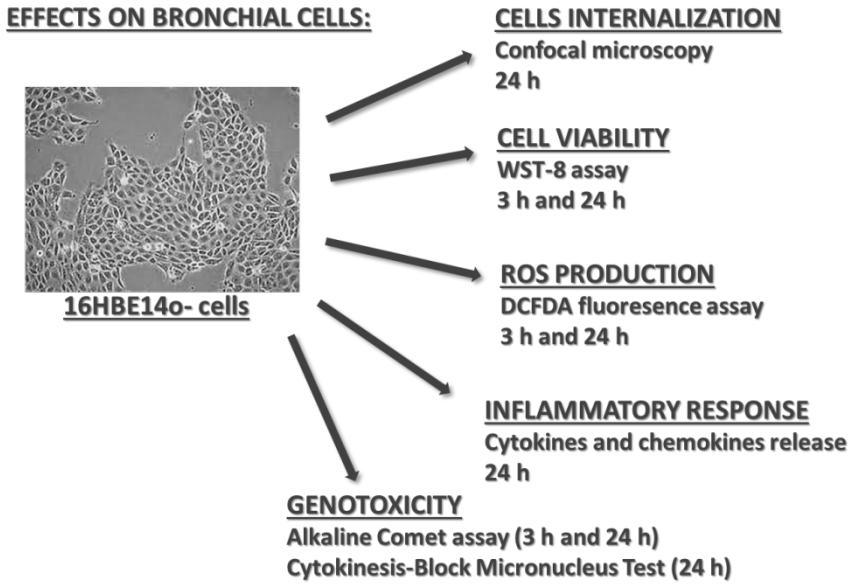


Figure 3. Scheme depicting the experimental design of the GrapHazard project. The effects of a panel of graphene-based materials differing by physico-chemical properties was evaluated on 16HBE14o− epithelial bronchial cells assessing different cellular parameters with a comparative approach.

The study was conducted with a comparative approach to define the role of selected GBM PC properties. In particular, for case-study 1, the effects of GO (reference material) were compared to those induced by rGO and wdGO to study the role of chemical reduction and formulation, respectively. For case-study 2, the effects of rGO1 (reference material) were compared to those of rGO2 – rGO4 to study the role of C/O ratio, to that of rGO5 to investigate the role of lateral dimension and to that of rGO6 to analyze the role of number of layers.

A detailed description of the methods used in the GrapHazard case-studies can be found in the corresponding publications (Pelin et al., 2023; Rodríguez-Garraus et al., 2023).

3.4 Toxicity assessment: secondary effects

Despite their high reproducibility, easy-to-use and convenience, traditional monocultures are unable to recapitulate *in vitro* the complex scenario of a target organ *in vivo*. For this reason, a wide range of advanced models has been set up, such as co- and tri-cultures models, 3D models, organoids, and others. These models are able to assess *in vitro* the occurrence of secondary toxic effects, that cannot be observed in monocultures. Secondary effects are cytotoxic responses observed in a certain target cell model as a consequence of the mediators released in the cellular environment by a second type of cells that is exposed to the test material. In GrapHazard, we planned to take advantage of two different co-culture models between bronchial cells (16HBE14o− cells) and macrophages (differentiated THP-1 cells; dTHP-1) to investigate secondary genotoxic and inflammatory effects induced by GBMs.

3.4.1 Cell lines

The 16HBE14o− cell line was cultured in standard conditions in a humidified incubator at 37 °C with 5% CO_2, as reported for the evaluation of primary effects.

THP-1 is a monocytic cell line isolated from peripheral blood of an acute monocytic leukemia patient. It has been extensively used to study monocyte/macrophage functions, mechanisms and signaling pathways, becoming a common model to estimate modulation of monocyte and macrophage activities. Similar to bronchial cells, THP-1 cells were cultured in standard conditions in a humidified incubator at 37 °C with 5% CO_2. Differentiation of THP-1 monocytes *in vitro* to obtain macrophages (dTHP-1) was achieved using 50 nM phorbol-12-myristate-13-acetate (PMA) for 24 h (Evans et al., 2019).

3.4.2 Co-culture approaches

In GrapHazard, two different co-culture systems were considered. The first one aimed at investigating the secondary genotoxic effects induced by GBM-treated macrophages in bronchial cells, according to the model established by (Evans et al., 2019). As shown in Figure 4, macrophages (dTHP-1) are co-cultured with the bronchial epithelial cells and treated with the tested material for 24 h. After that, the exposure medium is replaced with fresh medium with cytochalasin-B, to block cytokinesis during cell division, allowing the identification of cells that have divided once by their binucleated appearance. As both cell types are treated with the materials, the micronuclei scored in this approach could be induced by both primary effect of the material on the bronchial cells and secondary effects mediated by the macrophages. Therefore, the micronuclei rates should be compared with those obtained in the monoculture model.

The scoring of micronuclei should be restricted to the bronchial epithelial cells. Hence, an immunostaining technique is applied to differentiated dTHP-1 from the 16HBE14o- cells. Unfortunately, such differentiation could not be achieved during the timeline of GrapHazard, despite several fluorescent antibodies and experimental conditions were investigated. Hence, no results on the secondary genotoxic effects of GBMs could be obtained.

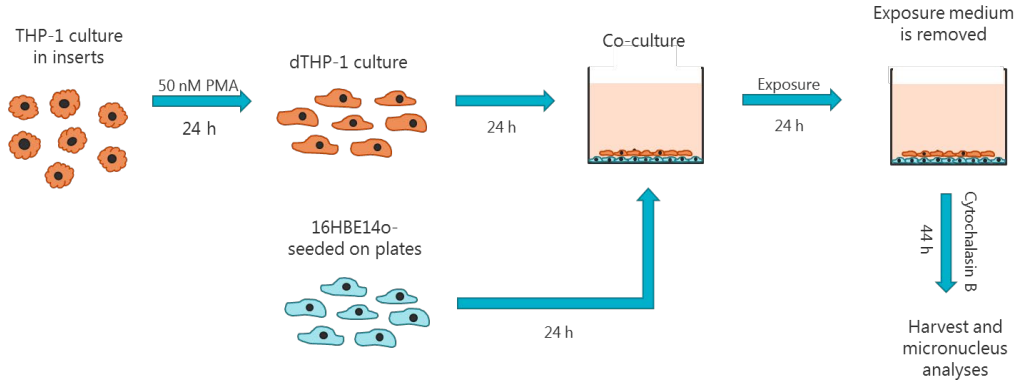


Figure 4. Experimental set up for the analysis of secondary genotoxic effects induced by graphene-based materials in bronchial cells.

The second co-culture system (Figure 5) was set up to investigate the secondary effects exerted by GBM-treated bronchial cells on macrophages, evaluating macrophages activation in the frame of an inflammatory response. In particular, bronchial cells were seeded in 24-well plates and exposed to each GBM (GO, rGO and wdGO) for 24 hours. After treatment, bronchial cells were co-cultured with macrophages probed with a

fluorescence dye and seeded in a Transwell™ system. Their activation was evaluated by means of cell migration after 4 h measuring the fluorescence signal given by migrated cells.

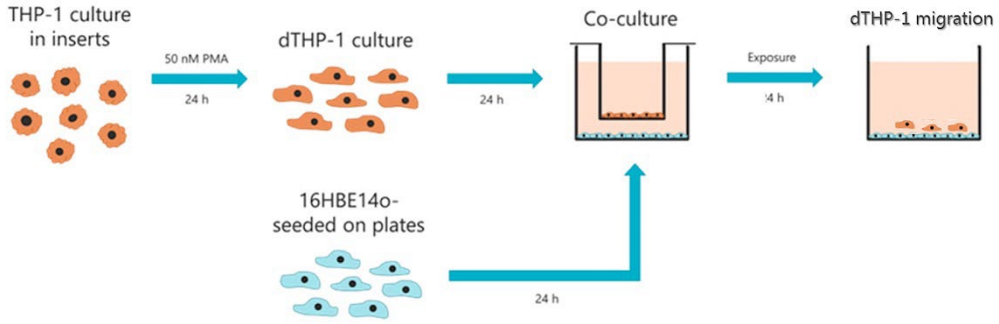


Figure 5. Experimental design for the evaluation of secondary inflammatory effects induced by graphene-based materials-treated bronchial cells in macrophages.

4 RESULTS AND DISCUSSION

4.1 Evaluation of primary effects induced in bronchial cells

4.1.1 Cellular uptake

Cellular internalization of each material was analyzed by laser confocal microscopy, exploiting light reflection properties of GBMs. Representative images of the case-study 1, reconstructed offline by merging red fluorescence (plasma membranes), blue fluorescence (nuclei) and green signal (light reflected by graphene derivatives), are shown in Figure 6. Images confirm the internalization of GO and rGO into bronchial cells, but not of wdGO. A similar pattern of cell interaction could be observed for GO and rGO, with most of the signals observed in the cytoplasm with varying degrees of patchiness. Furthermore, all the rGOs analyzed in the second case-study were efficiently internalized into bronchial cells (data not shown) and showed the same previous pattern.

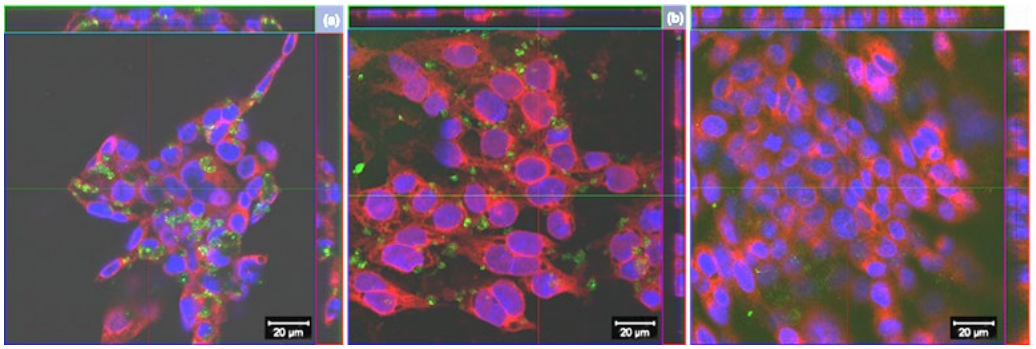


Figure 6. Orthogonal view of confocal images representing 16HBE14o− bronchial cells exposed to 25 µg/mL of (a) GO, (b) rGO or (c) wdGO for 24 h. Cell nuclei: blue; cell membranes: red; graphene materials: green. Images were captured with a confocal laser scanning microscope at a 40× magnification. Scale bar: 20 µm. Reprinted from (Pelin et al., 2023). Copyright 2023 by the authors.

4.1.2 Role of chemical reduction and formulation of graphene oxide

The main results related to case-study 1 are reported in a manuscript recently published in *Nanomaterials* (Pelin et al., 2023). Case-study 1 focused on the impact of two key factors on GO cytotoxic potential on bronchial cells: (i) its chemical reduction and (ii) its formulation as a stable dispersion. To this end, we evaluated the effects of GO in comparison to its chemically reduced form (rGO) to investigate the influence of chemical reduction and the effects of GO prepared as a powder or in a stable water dispersion

form to study the impact of formulation. All these materials were fully characterized under a physico-chemical point of view (refer to chapter 2.2) and tested by the TET assay to exclude any endotoxin contamination, which may be a confounding factor in the assessment of the cytotoxic effects of such materials. Physico-chemical analysis performed by TEM analysis demonstrated also that the tested materials were characterized by a similar lateral dimension profile: despite non-significant slightly different average dimensions, the materials were characterized by wide overlapping lateral dimension distributions that exclude any additional bias due to different sizes, an important property that may affect the cytotoxic potential of GO.

To characterize the hazard posed by the selected materials on bronchial cells, GBM cytotoxic potential on 16HBE14o− cells was investigated after a short (3 h) and a longer (24 h) exposure time by means of different cellular parameters: cell viability reduction (WST-8 reduction assay), ROS generation (DCFDA assay), inflammatory response (release of pro-inflammatory mediators) and genotoxicity, the latter evaluated as DNA and chromosome damage (Alkaline Comet assay and Cytokinesis-Block Micronucleus test, respectively). In particular, the WST-8 assay demonstrated that the three materials reduced cell viability with a significant different potency: GO induced a concentration-dependent reduction of cell viability with EC_{50} values of 56.4 µg/mL and 38.3 µg/mL after 3 and 24 h exposure, respectively; rGO induced a significantly higher effect, reducing cell viability with EC_{50} values of 16.23 µg/mL and 4.8 µg/mL after 3 and 24 h exposure, equal to 3.5-fold and 8-fold lower than those of GO, respectively. In contrast to GO, both 3 and 24 h 16HBE14o− cells exposure to wdGO did not reduce cell viability, but rather slightly increased it, suggesting a negligible cytotoxic potential. Considering that oxidative stress is proposed as a key mechanism involved in the toxicity of various nanomaterials (Könczöl et al., 2011; Lin et al., 2006; Yang et al., 2009), in the present study, GBMs were evaluated for their ability to increase ROS levels in 16HBE14o− cells up to 24 h exposure. Results confirmed the potency observed in the case of cell viability measurement, with rGO inducing a significantly higher ROS production than GO, despite being lower with respect to the positive control, suggesting a moderate oxidative stress potential. In addition, wdGO induced ROS production in a time- and concentration-dependent way, but its effect was slightly lower than that of GO, after 24 h exposure. Overall, these results (Figure 7) suggest the following rank of potency: rGO > GO > wdGO. The higher effect of rGO could be related to an enhanced physical–mechanical injury at the cellular level, consequent to rGO interaction with cell membranes. Indeed, as demonstrated by our results, rGO presented a wrinkled and twisted structure with sharp edges, whereas GO was characterized by smoother and rounded edges. In addition, strong conditions usually used in chemical methods employed in rGO production can influence its structure and its biological activity (Jarosz et al., 2016). In our study, we tested an rGO obtained by chemical reduction of GO using ascorbic acid which has been already

demonstrated to lead to less biocompatible rGO due to possible physical membrane damage induced by its irregular and wrinkled shape (Dervin et al., 2018). On the contrary, the lower cytotoxic potency of wdGO, especially in terms of lack of cell viability reduction, could be probably due to the stability of its dispersion, significantly higher than that of GO and rGO dispersions. Being the material in a stable dispersion, its deposition above cells and its subsequent interaction with cell membranes, appeared heavily limited, as suggested by the images acquired by confocal microscopy analysis, demonstrating that wdGO was the only material not interacting with cells nor being internalized. Hence, the consequent cell membranes physical disruption could be hampered, possibly explaining the lower cytotoxic effects in comparison to GO and rGO.

Given the ability of all the materials to increase ROS production in bronchial cells, we evaluated their effects by means of inflammatory response and genotoxicity, given their well-known correlation with oxidative stress (Kermanizadeh et al., 2015; Lugrin et al., 2014). Among the 12 evaluated pro-inflammatory mediators, a significant release in comparison to the untreated control was observed only for IL-1α, IL-6, IL-8 and TNF-α, suggesting a general low inflammatory potential. Considering IL-1α, as compared to the negative control (134 pg/mL), only rGO significantly increased its release to 1038 pg/mL (7.7-fold increase; $p < 0.0001$). Regarding TNF-α, rGO significantly increased its release (2721 pg/mL; 3.5-fold increase; $p < 0.01$) with respect to negative controls (770 pg/mL), with an effect higher with respect of that induced by GO (2045 pg/mL; 2.6-fold increase; $p < 0.05$). Regarding IL-6, as compared to the negative control (1624 pg/mL), only wdGO significantly increased its release to 3308 pg/mL (2-fold increase; $p < 0.05$). Similarly, wdGO was the only material able to significantly increase IL-8 release (4232 pg/mL; 2-fold increase; $p < 0.0001$) with respect to negative controls (2129 pg/mL). To identify any similarity between the pattern of pro-inflammatory release induced by GBM treatment with negative control, positive control or the reference material, the amount of each mediator (pg/mL) released in culture media by untreated cells, cells exposed to GO, rGO or wdGO, and those treated with the positive control (LPS) or the reference material (Mitsui-7 multi-wall carbon nanotubes; MWCNT), were displayed on a heatmap. A clustering analysis was performed, in which dendrograms represent the similarity between the different samples analyzed: the branch lengths are proportional to the similarities between samples, with the shorter branch indicating closer relationships. The clustering analysis performed on pro-inflammatory release data suggested for GO and rGO a cytokines release pattern with barely more similarities to those of the positive control and reference material than those of negative controls. On the contrary, wdGO appears to be the least inflammogenic material, showing a cytokines release pattern similar to that of negative controls (Figure 8).

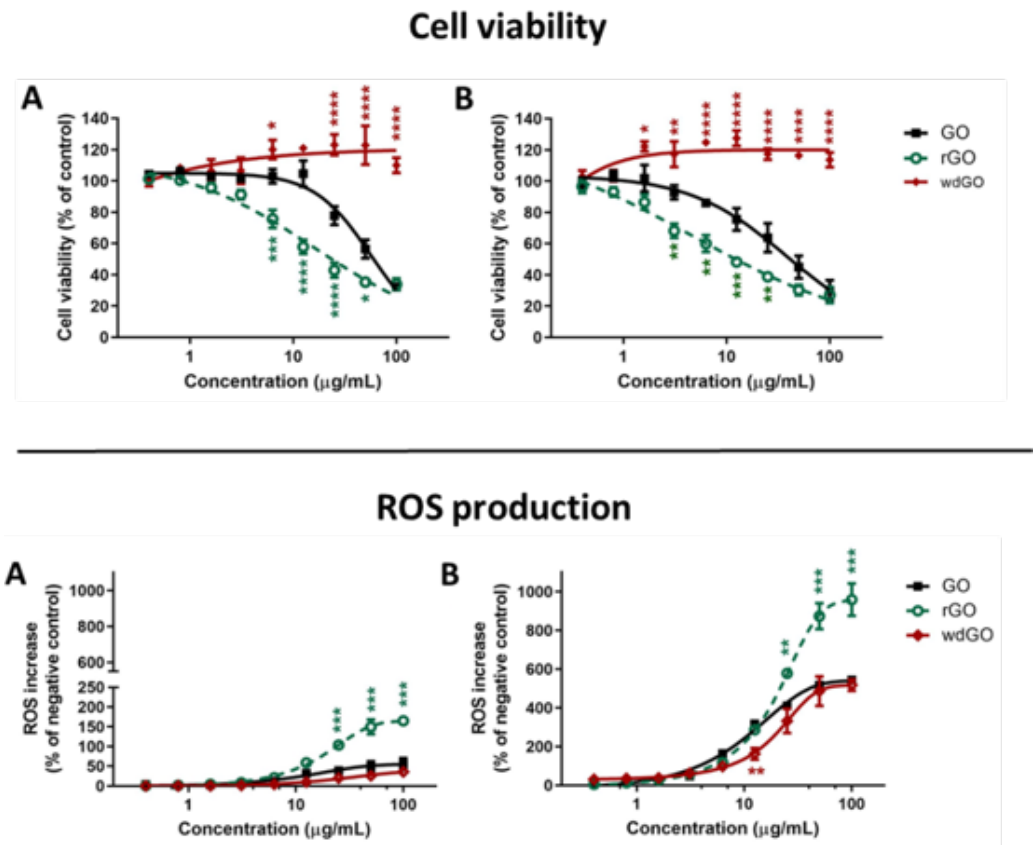
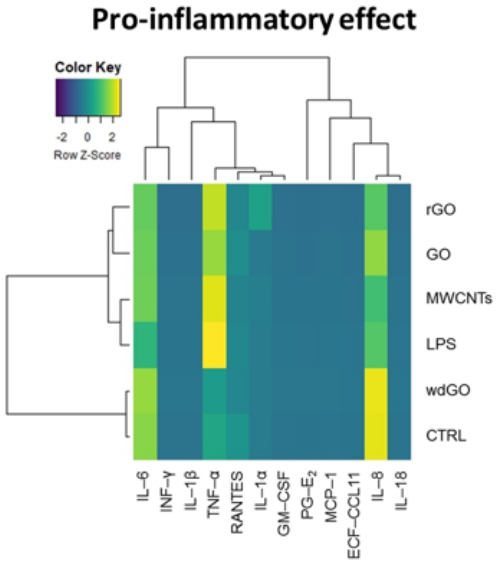


Figure 7. Role of chemical reduction and formulation of GO on its cytotoxic potential in 16HBE14o− bronchial cells. Cells viability (A) and reactive oxygen species (ROS) production (B) were evaluated by the WST-8 assay and DCFDA fluorescence probe, respectively, after 3 h (A) and 24 h (B) exposure. Data are reported as % of cell viability or ROS production in cells exposed to GBMs with respect to untreated control cells (negative control) and are the mean ± SE of three independent experiments performed in triplicate. Statistical differences vs. GO: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (two-way ANOVA and Bonferroni's post-test). Figure adapted from (Pelin et al., 2023).



Genotoxicity

	DNA DAMAGE		MICRONUCLEI INDUCTION
	3 h	24 h	
GO	no	no	no*
rGO	no	no*	no
wdGO	no	no	n0

** A linear concentration-dependent response was observed*

Figure 8. Pro-inflammatory effects induced by GO, rGO or wdGO in bronchial cells, evaluated measuring the release from 16HBE14o− cells of 12 inflammatory mediators after 24 h exposure to each GBM (10 µg/mL). Data are visualized in a heatmap on which a clustering analysis was performed to identify any similarities with the positive control LPS (1 µg/mL) and multi-walled carbon nanotubes (MWCNTs) Mitsui-7 (1 µg/mL) as reference material. Genotoxic effect induced by GO, rGO or wdGO in bronchial cells, were evaluated by means of induction of DNA damage in 16HBE14o− cells through the alkaline comet assay after 3 and 24 h treatment as well as by means of micronuclei induction through the Cytokinesis-Block Micronucleus test after 24 h exposure. Figure adapted from (Pelin et al., 2023).

Regarding the genotoxic effects (Figure 8), GO, rGO and wdGO were unable to significantly increase the frequency of DNA damage, as compared to the negative control, although a significant linear concentration-dependent response was observed for rGO ($p < 0.005$, slope = 0.129) after 24 h exposure. Similarly, none of the materials induced a significant increase in the frequency of micronuclei compared with the negative control at any of the tested concentrations, although a significant linear concentration-dependent response ($p < 0.005$, slope = 0.086) was found only for GO. We should emphasize that the lack of genotoxic potential should be considered in view of materials cells internalization studies performed by confocal microscopy analysis in reflection mode, showing that GO and rGO, but not wdGO, are indeed retained into cells. Therefore, we can suggest that the lack of genotoxicity in bronchial cells is not a result of

a simple inability of the materials to reach nucleic acids, but an intrinsic feature of the materials themselves.

Overall, our results suggest that rGO is the most cytotoxic material for epithelial bronchial cells among the tested GO derivatives, particularly in terms of cell viability reduction and increased ROS production. The increased cytotoxicity of rGO, as compared to that of GO, could be due to its chemical reduction, probably inducing material structure alterations such as sharp edges and wrinkled structures leading to membrane damages. As a second result, this study indicates that GO formulated in a stable water dispersion form is highly biocompatible, probably reducing its mechano–physical interaction with cell membranes, leading to cell damage. These results acquire a significant importance for physicists, chemists and materials scientists specialized in the field of GBMs, given that dispersed and powdered GO have applications in different technological fields.

4.1.3 Role of oxidation status, number of layers and lateral size of reduced graphene oxide

The main results related to case-study 2 are reported in a manuscript that has been accepted for publication in *Nanotoxicology* (Rodríguez-Garraus et al., 2023). Case-study 2 focused on the influence of three important PC properties – oxidation status, lateral size, and number of layers – on the cytotoxic and genotoxic potential exerted by rGO. Of the six materials that were analyzed, rGO1-rGO4 only differed in the carbon-to-oxygen (C/O) content, whereas rGO5 and rGO6 were characterized by different lateral size and number of layers, respectively, but similar C/O content compared with that of rGO1. In the same way to the previous case-study, the rGOs were thoroughly characterized and the lack of endotoxin contamination was confirmed for all the materials. As shown in Table 1, the C/O content decreased from rGO1 to rGO4, meaning that rGO4 had the highest content of oxygen groups. On the other hand, rGO5 had the smallest lateral size (< 1 µm) compared to the other rGOs (~40 µm); while rGO6 showed double the number of layers than the other materials.

As described in the previous section regarding the first case-study, the toxicological endpoints evaluated in the bronchial cells after a short (3 h) and a longer (24 h) treatment were: cell viability (assessed by the the ATP-luminometric and the WST-8 assays), generation of ROS (DCFDA assay), inflammatory response (by the release of pro-inflammatory mediators) and the induction of DNA and chromosome damage (Alkaline Comet assay and Cytokinesis-Block Micronucleus test, respectively). The impact of C/O ratio on cytotoxicity and ROS production was evaluated by comparing rGO1, rGO2, rGO3 and rGO4. Then, the influence of lateral size and number of layers was assessed by comparing rGO1 with rGO5, and rGO6, respectively.

The results of the colorimetric WST-8 assay, which were similar to those obtained by the luminescence assay, are shown in Figure 9. The EC_{50} values after 3 h exposure to the rGOs were higher than 100 µg/mL for rGO1, rGO4 and rGO5, whereas values of 90.4 and 50.4 µg/mL were observed for rGO2 and rGO3, and rGO6 showed no reduction in cell viability. The corresponding values were 69.9, 14.2, 4.6, 16.7, 78.1 and > 100 µg/mL after 24 h exposure to rGO1-rGO6. These findings suggested the following cytotoxicity potency rank: rGO3 (11.0 C/O content) > rGO2 (13.9) > rGO4 (7.1) > rGO1 (52.5). Hence, although rGOs characterized by higher densities of oxygen content seemed to be more cytotoxic than rGO1, a direct correlation with the amount of oxygen groups could not be established, probably due to the small variation in the C/O content of rGO2 – rGO4 (Table 1). These results agree with those previously reported by Chatterjee and colleagues (Chatterjee et al., 2014), who observed a similar cytotoxic response by two materials (GO and rGO) with similar lateral size, thickness, and layer number but different oxidation state. No differences were observed in the cytotoxic potency between rGO5 and rGO1, suggesting a lack of influence by the lateral dimension. On the other hand, the increased number of flakes of rGO6, compared to rGO1, seemed to be associated with a reduction in the cytotoxic potential.

The ability of the rGOs to induce the formation of ROS was evaluated by exposing 16HBE14o− cells to the materials up to 24 h (Figure 10). After 3 h exposure, the effects exerted by rGO2, rGO3 and rGO4 were slightly higher, although not statistically significant, than that of rGO1. This tendency continued up to 24 h exposure, when a similar potency in the induction of ROS was observed for the four rGOs. Hence, the differences in C/O content did not influence the ROS generation capacity of the rGOs. These results disagree with those reported by Majeed and colleagues (Majeed et al., 2017), who observed an increased ROS induction associated with a higher degree of oxidation. Regarding the lateral size, no significant differences were observed between rGO5 and rGO1 in ROS production at both exposure times, indicating a lack of influence of this parameter. On the other hand, in agreement with the outcomes of the cell survival assessment, rGO6 induced ROS production at much lower potency than rGO1, both after 3 and 24 h exposures. These findings suggest that a higher number of layers may reduce the ROS induction potential of rGO.

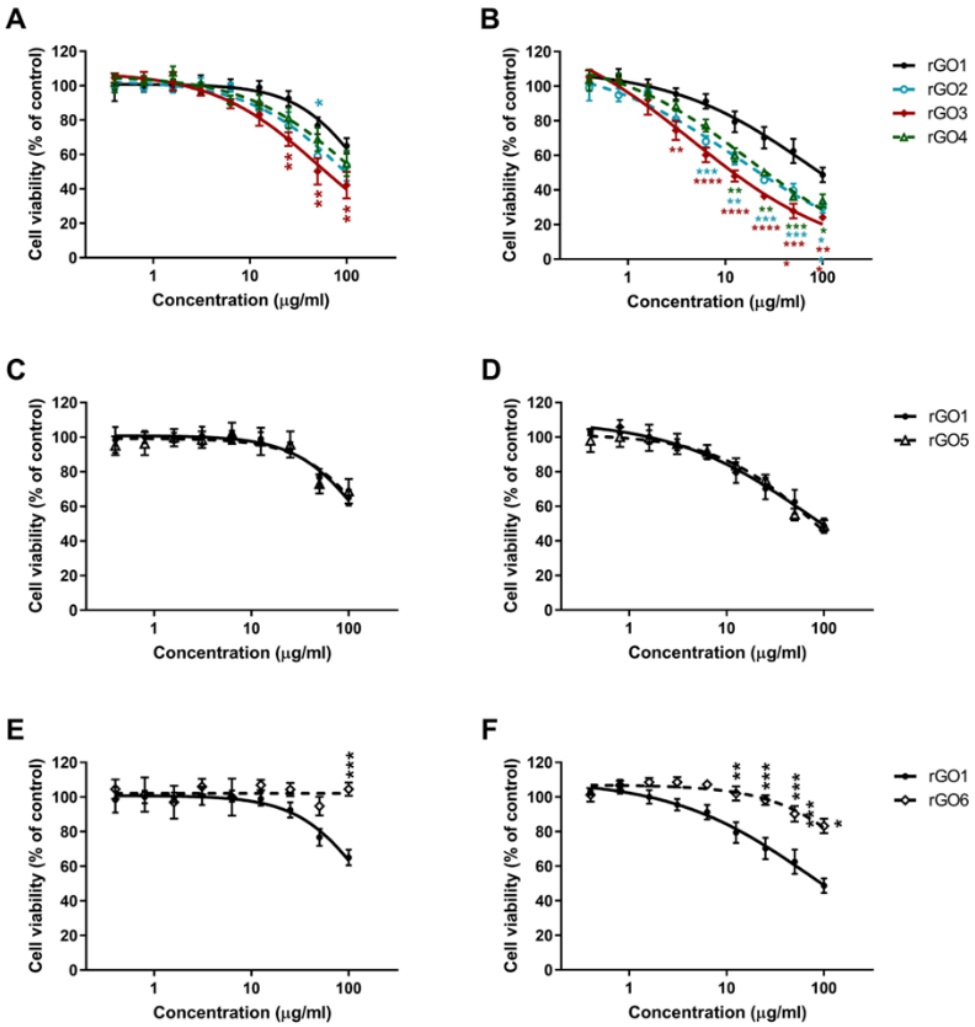


Figure 9. Effects of rGO1 – rGO6 on 16HBE14o– cells viability evaluated by the WST-8 assay after 3 h (A, C, E) and 24 h (B, D, F) exposure. Data are reported as % of cell viability in cells exposed to rGOs with respect to untreated control cells (negative control) and represented as the mean ± SE of 3 independent experiments performed in triplicate. Statistical differences vs rGO1: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (Two-way ANOVA and Bonferroni's post-test). Reprinted from (Rodríguez-Garraus et al., 2023). Copyright 2023 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.

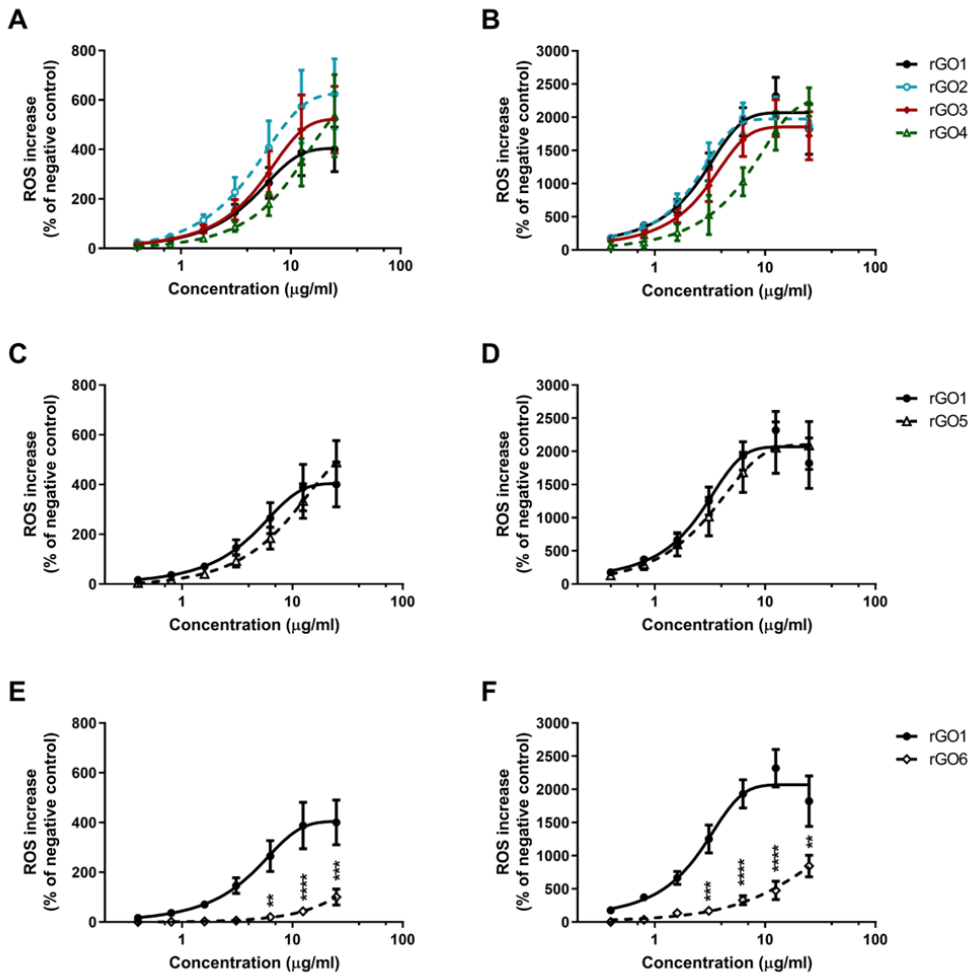


Figure 10. Reactive oxygen species (ROS) production in 16HBE14o− cells after exposure to rGO1 − rGO6 for 3 h (A, C, E) or 24 h (B, D, F), evaluated by the DCFDA assay. Results are expressed as % of ROS increase with respect to negative control (cells not exposed to rGOs) and represented as the mean ± SE of at least 3 independent experiments performed in triplicate. Statistical differences vs rGO1: ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (two-way ANOVA and Bonferroni's post-test). Reprinted from (Rodríguez-Garraus et al., 2023). Copyright 2023 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.

As in the first case-study, the inflammogenic and genotoxic potential of the rGOs was also evaluated, and the results are summarized in Table 2. Only 4 out of a panel of 12 pro-inflammatory mediators (IL-1α, IL-1β, IL-6, IL-8, IL-18, TNF-α, GM-CSF, INF-γ, MCP-1, RANTES, ECF/CCL11, PG-E2) showed a significant effect after 24 h exposure to rGOs. An increase of the oxygen content seemed to enhance the release of IL-1α, as rGO2-rGO4 showed a significant increased release of this cytokine compared to rGO1. On the other hand, rGO3 and rGO4 induced a significant increase of IL-6 with respect to the negative control, but not different than the release induced by rGO1. As compared to rGO1, rGO3 also induced a significant increased GM-CSF release. Furthermore, the material with the smallest lateral size, rGO5, induced a significant decrease of TNF-α release compared with rGO1, but none of the materials significant differ from the negative control. These findings agree with those reported by Li and colleagues (Li et al., 2018), who observed a correlation between the oxidation status and the induction of lung inflammation after evaluating GO and rGOs with very similar number of layers and lateral size.

Among the rGOs analyzed in the present study, only those with the lowest oxygen content were able to induce genotoxic effects (Table 2). After a short exposure time (3 h), none of the rGOs was able to induce DNA alterations, while a dose-dependent increase of DNA damage was induced by rGO1 after 24 h exposure. On the other hand, rGO1, rGO5 and rGO6 were able to induce chromosome damage, assessed by the micronucleus assay, in a dose-dependent manner. It is worth noting that the negative outcomes cannot be attributed to a lack of interaction of the rGOs with the cellular genetic material as all of them were efficiently internalized by the cells. Negative genotoxic outcomes have also been previously reported for other rGOs characterized by a similar C/O ratio as rGO2-rGO4 (Bengtson et al., 2016; Cebadero-Dominguez et al., 2023), but different lateral size and number of layers than the ones analyzed in the present study.

In summary, a higher oxygen content of rGOs seems to be barely associated with a higher cytotoxic and early ROS-inducing potential. On the other hand, genotoxic effects were observed with the rGO of lowest density of oxygen groups. However, direct correlation between the C/O content cannot be established for any of the hazard endpoints. On the other hand, increasing number of layers may contribute to a decreased potential for inducing cytotoxicity and ROS production, whereas no influence of the lateral size was observed.

Table 2. Pro-inflammatory and genotoxic potential of reduced graphene oxides

Pro-inflammatory response*		
IL-1α		rGO2-rGO4 > rGO1
IL-6		No significant differences with rGO1
GM-CSF		rGO3 > rGO1
TNF-α		rGO5 < rGO1
Genotoxicity		
DNA damage	3 h	Significant effects induced by none of the rGOs
	24 h	rGO1 induced a significant dose-dependent increase
Micronuclei induction		rGO1, rGO5 and rGO6 induced a significant dose-dependent increase

* No significant effects observed for IL-1β, IL-8, IL-18, INF-γ, MCP-1, RANTES, ECF/CCL11 and PG-E2.

4.2 Secondary effects induced by graphene-based materials

A co-culture system was set up to investigate the secondary effects exerted by GBM-treated bronchial cells on macrophages, evaluating the activation of the latter in the frame of an inflammatory response. To this aim, the materials described in the first case-study (GO, rGO and wdGO), were tested, since among these materials the difference in terms of cytotoxicity potency and inflammatory reaction were particularly evident, as shown in chapter 4.1.2. In particular, after 24 h exposure to each GBM (0.01 – 100 µg/mL), bronchial cells were co-cultured with dTHP-1 in a Transwell™ system to measure macrophages migration after 4 h of co-culturing. As depicted in Table 3, all materials slightly, but significantly, increase macrophages migration over negative controls. The effect was observed up to the concentration of 1 µg/mL, since at higher concentrations migration measurement was affected by the significant reduction of cell viability induced in both bronchial cells and macrophages, at least by GO and rGO. Notwithstanding, even though all the materials slightly increase macrophages migration through bronchial cells activation, differences in their potencies were barely detectable: indeed, GO and rGO induced a similar effect, whereas wdGO potency appeared to be slightly lower. In any case, it should be noted that these effects were low, also comparing that induced by the positive control MIP-3α (1.25×10^{-12} M), a well-known chemokine. On the whole, this result, together with the low amount of chemokines released by bronchial cells after 24 h

exposure to each material, suggests a very low pro-inflammatory potential, not only considering a primary, but also a secondary inflammatory response. Indeed, among a panel of chemokines [IL -8, monocyte chemoattractant protein-1 (MCP-1), eosinophil chemotactic factor (ECF/CCL11), regulated upon activation normal T cell expressed and secreted (RANTES)], only IL-8 was significantly increased by bronchial cells treatment with the selected GBMs (10 µg/mL) after 24 h, and in particular only by wdGO that, however, considering the whole pattern of pro-inflammatory mediators measured, appeared to be the less inflammogenic material (see chapter 4.1.2). Therefore, these results suggest that these materials are able to only barely induce a slight primary inflammatory reaction in bronchial cells, that, in turn, is able to modulate a modest and slight secondary inflammatory reaction in macrophages, highlighting a general low inflammogenic potential.

Table 3. Secondary inflammatory effects induced in macrophages by GBM-treated bronchial cells for 24 h. Secondary effects were evaluated by means of macrophages migration (-: no effect; +: migration > 5% of negative controls; ++: migration > 10% of negative controls; +++: migration > 25% of negative controls) after their co-culture with GBM-treated bronchial cells for 4 h. Primary cytotoxicity induced by 24 h exposure to each GBM in bronchial cells and macrophages was evaluated by means of cell reduction through the WST-8 assay. Chemokines release (IL-8, MCP-1, ECF/CCLL11 and RANTES) was evaluated after 24 h exposure to each GBM (10 µg/mL) after 24 h by ELISA assays.

	Secondary macrophages migration	Primary macrophages cytotoxicity	Primary bronchial cells cytotoxicity	Chemokines release from bronchial cells
Powdered graphene oxide (GO)				-
100 µg/mL	-	> 50%	> 50%	
10 µg/mL	-	> 25%	> 25%	
1 µg/mL	++	-	-	
0.1 µg/mL	++	-	-	
0.01 µg/mL	++	-	-	

Table 3 (Cont.)

	Secondary macrophages migration	Primary macrophages cytotoxicity	Primary bronchial cells cytotoxicity	Chemokines release from bronchial cells
Reduced graphene oxide (rGO)				-
100 µg/mL	-	> 50%	> 50%	
10 µg/mL	-	> 50%	> 50%	
1 µg/mL	++	-	-	
0.1 µg/mL	+	-	-	
0.01 µg/mL	+	-	-	
Water-based dispersed graphene oxide (wdGO)				-*
100 µg/mL	-	-	-	
10 µg/mL	-	-	-	
1 µg/mL	+	-	-	
0.1 µg/mL	-	-	-	
0.01 µg/mL	-	-	-	
MIP-3α				
1.25 x 10^{-12} M	+++	-	-	N/A

N/A: not available

* Only IL-8 was significantly increased.

5 RECOMMENDATIONS FOR A SAFE USE OF GBMS IN OCCUPATIONAL SETTINGS

In connection with task 6 (*Correlations between in vitro toxicity data and human data from workers exposed to GBMs*), a collaboration between GrapHazard and the FIOH's team performing occupational studies within the European Graphene Flagship (Grant agreement ID: 881603) was established. The original idea was to correlate the toxicity data obtained in GrapHazard with data from human biomonitoring studies that were planned to be done within the Graphene Flagship project. Unfortunately, no human biomonitoring studies could finally be performed within the Flagship due to several reasons (i.e., low number of workers per company, pandemic limitations, etc). Nevertheless, exposure measurements and occupational hygiene assessment were conducted in several laboratories and companies working with GBMs.

In occupational settings, worker exposure to GBMs is related to the processes and activities during the synthesis and manufacturing stages of products. The final stages of synthesis/production process, when the raw material is dried and packed for further use, are the most critical points regarding the workers' exposure; in addition to the maintenance and cleaning tasks of the process equipment, where dry material can be released uncontrollably/accidentally. Besides, occupational exposure may potentially be significant at the end-of-life scenarios, such as recycling and waste handling.

Within the Graphene Flagship, the occupational exposure to possibly released graphene nanoparticles in air was assessed during graphene related work operations, according to EN standards (EN 16966:2018; EN 17058:2018). A total of seven different exposure scenarios were assessed in five workplaces: two commercial companies producing graphene, GO and rGO, and three research institutions (performing tasks at pilot and laboratory scale) working with GO, rGO and other types of GBMs. Figure 11 illustrates some of these scenarios. The outcomes of the study showed low exposure levels and risk during the production of GBMs and related activities. This was probably due to the good level of awareness about the possible health risks associated with GBMs' exposure in the studied workplaces, where the implementation of safety measures and practices for protecting workers was good.

Very limited information is currently available on airborne concentrations and human biomonitoring data related to GBMs exposure in occupational settings. Increased particle number concentrations were reported during the production of graphene (Lee et al., 2016), few-layer graphene (Boccuni et al., 2020; Tombolini et al., 2021) and graphene nanoplatelets (Bellagamba et al., 2020; Bellagamba et al., 2023). However, the

composition of the air particles was not determined in most of these studies. As concerns human toxicity data, a study performed in a laboratory during the production process of FLG by liquid-phase exfoliation observed no differences between a small group of workers (n= 6) and controls (n= 11) in the levels of oxidative stress and inflammatory biomarkers (Ursini et al., 2021). There was an increase, although non-significant, of the frequency of micronuclei in buccal cells of workers in respect to controls, and a significant increase of oxidative DNA damage in lymphocytes. Interestingly, both genotoxicity biomarkers showed a reduction in a follow-up study for the same population conducted six months after installing a filter hood (Cavallo et al., 2022).

Figure 11. A worker emptying and cleaning a dust drum (left) containing graphene powder (right). Courtesy of Tomi Kanerva (Finnish Institute of Occupational Health).

No official occupational exposure limit (OEL) values currently exist for graphene nanomaterials. Based on a subchronic inhalation study in rats and using the multi-path particle dosimetry model to estimate the deposition fraction in the human alveolar region, Lee and colleagues (Lee et al., 2019) derived an OEL value of 18 μg/m^3 for graphene. On the other hand, although nano reference values 8 h time-weighted average have been proposed for some nanoparticles, they are not recommended for non-spherical materials as GBMs.

Based on the performed exposure assessment and the hazard information collected in GrapHazard and available in the literature, the Finnish Institute of Occupational Health (FIOH) has edited a factsheet with recommendations to workers of the GBMs' manufacturing sector. The factsheet is available in English, Finnish, Italian and Spanish languages; and it can be downloaded from the GrapHazard webpage at FIOH (Hazard characterization of graphene based nanomaterials in energy production and storage(GrapHazard)– SAF€RA | Finnish Institute of Occupational Health (ttl.fi)).

6 DISSEMINATION

The project has been extensively disseminated through newsletters and social media, and a dedicated webpage was created at the FIOH' website (https://www.ttl.fi/en/research/projects/hazard-characterization-of-graphene-based-nanomaterials-in-energy-production-and-storagegraphazard). In addition, the results of the project have also been made publicly available through the SAF€RA partnership (https://www.safera.eu).

A factsheet on "Best practices for safe graphene work" edited by FIOH and available in 4 different languages can be downloaded from the GrapHazard webpage (see chapter 5).

Peer-reviewed publications:

- Domenech et al. (2022) Genotoxicity of Graphene-based materials. *Nanomaterials*, 12, 1795 (openly available at https://www.mdpi.com/2079-4991/12/11/1795).
- Pelin et al. (2023) Role of Chemical Reduction and Formulation of Graphene Oxide on Its Cytotoxicity towards Human Epithelial Bronchial Cells. *Nanomaterials*, 13, 2189 (openly available at https://www.mdpi.com/2079-4991/13/15/2189).
- Rodríguez-Garraus et al. Impact of physico-chemical properties on the toxicological potential of reduced graphene oxide in human bronchial epithelial cells (*Nanotoxicology*, in press).

Conferences and seminars:

- Catalán and Pelin. Updates of the GrapHazard project. SAF€RA symposia (Rome, May 2022 and Toulouse, May 2023).
- Rodríguez-Garraus et al. Role of carbon-to-oxygen ratio on the cytotoxicity and genotoxicity of reduced graphene oxide towards human bronchial cells. "Nano-week" & NanoCommons Final Conference (Cyprus, 20-24 June 2022).
- Carlin et al. Reduction of graphene oxide increases its pro-inflammatory effect on bronchial epithelial cells. "Nano-week" & NanoCommons Final Conference (Cyprus, 20-24 June 2022).
- Carlin et al. Reduction and formulation of graphene oxide modulate its cytotoxicity towards bronchial cells. The XVIth International Congress of Toxicology (ICT2022, Maastricht, 18-21 September 2022).
- Aimonen & Poikkimäki. Grafeenin turvallisuustutkimus Työterveyslaitoksella. Nanoteknologia EU:n vihreän ja digitaalisen siirtymän tukena (Webinar, 11 October 2022). Organized by the Finnish Ministry of Social Affairs and Health.

- Catalán. FIOH's involvement into nano-related projects & initiatives. Nordic Nano Group meeting (Helsinki, 26 October 2022). Organized by the Finnish Safety and Chemicals Agency.
- Carlin et al. Pro-inflammatory effects of graphene oxide and reduced graphene oxide on bronchial epithelial cells. 41th Congress of the Italian Society of Pharmacology (Rome, 16-19 November 2022).
- Passerino et al. Effetti *in vitro* del grafene ossido sulle cellule epiteliali bronchiali umane: influenza della formulazione e della riduzione chimica. 21st Congress of the Italian Society of Toxicology (Bologna, 20-22 February 2023).
- Rodríguez-Garraus et al. Effect of the oxidation status of reduced graphene oxide on the genotoxicity of human bronchial cells. 51st European Environmental Mutagenesis and Genomics Society (EEMGS) & 27th Spanish Environmental Mutagenesis and Genomics Society (SEMA) meeting (Málaga, 15-18 May 2023).
- Venäläinen et al. Assessment of primary and secondary genotoxicity of reduced graphene oxide using an *in vitro* lung co-culture model. 8th International Conference on Environmental, Health and Safety issues related to Nanomaterials, nanoSAFE'23 and NanoSafety Cluster joint conference (Grenoble, 5-9 June 2023).
- Pelin. Hazard characterization of graphene-related materials: an integrated approach for the assessment of their safety. 2nd Annual Conference on Global Nanotechnology (NanoSeries2023) (Madrid, 19-21 June 2023).

7 CONCLUSIONS

In conclusion, our findings suggest that the toxicity assays that have been adapted for the hazard characterization of nanomaterials can successfully be used for GBMs as far as the potential sources of interferences are properly assessed and controlled. The cytotoxic and genotoxic potential of GBMs towards bronchio-epithelial cells depends on the production process and the PC properties of the materials. The reduction process applied to GO to obtain rGO seems to induce a reduction of cell viability and an increase in the generation of ROS when the cells are exposed to these materials. On the other hand, a well-dispersed and stable water-based formulation of GO appears to prevent the material to be internalized into the cells and exert the cytotoxic effects observed with the same powdered GO. These results acquire a significant importance, given that dispersed and powdered GO have applications in different technological fields. As regards the main PC parameters that define the GBMs family, the results of the present study with different rGOs indicate that, although it is not possible to establish a direct correlation between the oxidation status of the materials and any of the hazard endpoints that were assessed, there seems to be an association of higher oxygen content with higher cytotoxicity and early ROS induction, whereas genotoxic effects were observed with the rGO of lowest density of oxygen groups. On the other hand, the potential for inducting cytotoxicity and ROS production may be decreased by increasing the number of layers of the rGOs, whereas the lateral size does not seem to have any influence. The observed results can be of interest when considering the safe-by-design production of new rGOs.

Despite inhalation being the main exposure route to GBMs in occupational settings, limited information on airborne GBM concentrations and human biomonitoring data is currently available. Therefore, minimization of exposure to GBMs by applying the most appropriate occupational hygiene measures is highly recommended.

8 ACKNOWLEDGEMENTS

This project was financed by the Finnish Work Environment Fund (project number 200338), the Italian National Institute for Insurance against Accidents at Work (INAIL), the Finnish Institute of Occupational Health and the University of Trieste, in the frame of the SAF€RA 2020 program. The authors are grateful to these organizations for providing the financial support and being part of the Advisory Committee of the project.

The graphene manufacturing companies Graphenea SA, Avanzare Innovación Tecnológica SL, Grupo Antolín SA and SixoniaTech GmbH are strongly acknowledged for having provided the tested materials and characterization information, and for their useful advice as members of the Advisory Committee.

The authors would also like to thank Mira Hartikainen for excellent technical support, and Outi Kulo and Reija Rahunen for helpful project assistance. Tomi Kanerva is very much acknowledged for sharing the outcomes and images of the studies performed by the Finnish Institute of Occupational Health within the EU Graphene framework.

LITERATURE

Achawi, S., Pourchez, J., Feneon, B., & Forest, V. (2021). Graphene-Based Materials in Vitro Toxicity and Their Structure-Activity Relationships: A Systematic Literature Review. In *Chemical Research in Toxicology* (Vol. 34, pp. 2003-2018): American Chemical Society.

Aimonen, K., Suhonen, S., Hartikainen, M., Lopes, V. R., Norppa, H., Ferraz, N., & Catalán, J. (2021). Role of Surface Chemistry in the In Vitro Lung Response to Nanofibrillated Cellulose. *Nanomaterials (Basel)*, *11*(2). https://doi.org/10.3390/nano11020389

Akhavan, O., Ghaderi, E., & Akhavan, A. (2012). Size-dependent genotoxicity of graphene nanoplatelets in human stem cells. *Biomaterials*, *33*(32), 8017-8025. https://doi.org/10.1016/j.biomaterials.2012.07.040

Alwarappan, S., Erdem, A., Liu, C., & Li, C.-Z. (2009). Probing the Electrochemical Properties of Graphene Nanosheets for Biosensing Applications. *The Journal of Physical Chemistry C*, *113*(20), 8853-8857. https://doi.org/10.1021/jp9010313

Balandin, A. A., Ghosh, S., Bao, W., Calizo, I., Teweldebrhan, D., Miao, F., & Lau, C. N. (2008). Superior Thermal Conductivity of Single-Layer Graphene. *Nano Letters*, *8*(3), 902-907. https://doi.org/10.1021/nl0731872

Bellagamba, I., Boccuni, F., Ferrante, R., Tombolini, F., Marra, F., Sarto, M. S., & Iavicoli, S. (2020). Workers' Exposure Assessment during the Production of Graphene Nanoplatelets in R&D Laboratory. *Nanomaterials*, *10*(8), 1520. https://www.mdpi.com/2079-4991/10/8/1520

Bellagamba, I., Boccuni, F., Ferrante, R., Tombolini, F., Natale, C., Marra, F., Sarto, M. S., & Iavicoli, S. (2023). Occupational Exposure during the Production and the Spray Deposition of Graphene Nanoplatelets-Based Polymeric Coatings. *Nanomaterials*, *13*(8), 1378. https://www.mdpi.com/2079-4991/13/8/1378

Bengtson, S., Kling, K., Madsen, A. M., Noergaard, A. W., Jacobsen, N. R., Clausen, P. A., Alonso, B., Pesquera, A., Zurutuza, A., Ramos, R., Okuno, H., Dijon, J., Wallin, H., & Vogel, U. (2016). No cytotoxicity or genotoxicity of graphene and graphene oxide in murine lung epithelial FE1 cells in vitro. *Environmental and Molecular Mutagenesis*, *57*(6), 469-482. https://doi.org/10.1002/em.22017

Bianco, A. (2013). Graphene: Safe or Toxic? The Two Faces of the Medal. *Angewandte Chemie International Edition*, *52*(19), 4986-4997. https://doi.org/https://doi.org/10.1002/anie.201209099

Bianco, A., Cheng, H.-M., Enoki, T., Gogotsi, Y., Hurt, R. H., Koratkar, N., Kyotani, T., Monthioux, M., Park, C. R., Tascon, J. M. D., & Zhang, J. (2013). All in the graphene family – A recommended nomenclature for two-dimensional carbon materials. *Carbon*, *65*, 1-6. https://doi.org/10.1016/j.carbon.2013.08.038

Boccuni, F., Ferrante, R., Tombolini, F., Natale, C., Gordiani, A., Sabella, S., & Iavicoli, S. (2020). Occupational exposure to graphene and silica nanoparticles. Part I: workplace measurements and samplings. *Nanotoxicology*, *14*(9), 1280-1300. https://doi.org/10.1080/17435390.2020.1834634

Brownson, D. A. C., Kampouris, D. K., & Banks, C. E. (2011). An overview of graphene in energy production and storage applications. *Journal of Power Sources*, *196*(11), 4873-4885. https://doi.org/https://doi.org/10.1016/j.jpowsour.2011.02.022

Callaghan, Patrick J., Ferrick, B., Rybakovsky, E., Thomas, S., & Mullin, James M. (2020). Epithelial barrier function properties of the 16HBE14o- human bronchial epithelial cell culture model. *Bioscience Reports*, *40*(10). https://doi.org/10.1042/bsr20201532

Cavallo, D., Ursini, C. L., Fresegna, A. M., Ciervo, A., Boccuni, F., Ferrante, R., Tombolini, F., Maiello, R., Chiarella, P., Buresti, G., Del Frate, V., Poli, D., Andreoli, R., Di Cristo, L., Sabella, S., & Iavicoli, S. (2022). A follow-up study on workers involved in the graphene production process after the introduction of exposure mitigation measures: evaluation of genotoxic and oxidative effects. *Nanotoxicology*, *16*(6-8), 776-790. https://doi.org/10.1080/17435390.2022.2149359

Cebadero-Dominguez, Ó., Medrano-Padial, C., Puerto, M., Sánchez-Ballester, S., Cameán, A. M., & Jos, Á. (2023). Genotoxicity evaluation of graphene derivatives by a battery of in vitro assays. *Chemico-Biological Interactions*, *372*. https://doi.org/10.1016/j.cbi.2023.110367

Chatterjee, N., Eom, H. J., & Choi, J. (2014). A systems toxicology approach to the surface functionality control of graphene-cell interactions. *Biomaterials*, *35*(4), 1109-1127. https://doi.org/10.1016/j.biomaterials.2013.09.108

Chortarea, S., Kuru, O. C., Netkueakul, W., Pelin, M., Keshavan, S., Song, Z., Ma, B., Gómes, J., Abalos, E. V., Luna, L. A. V. d., Loret, T., Fordham, A., Drummond, M., Kontis, N., Anagnostopoulos, G., Paterakis, G., Cataldi, P., Tubaro, A., Galiotis, C., Kinloch, I., Fadeel, B., Bussy, C., Kostarelos, K., Buerki-Thurnherr, T., Prato, M., Bianco, A., & Wick, P. (2022). Hazard assessment of abraded thermoplastic composites reinforced with reduced graphene oxide. *Journal of Hazardous Materials*, *435*, 129053. https://doi.org/https://doi.org/10.1016/j.jhazmat.2022.129053

Dervin, S., Murphy, J., Aviles, R., Pillai, S. C., & Garvey, M. (2018). An in vitro cytotoxicity assessment of graphene nanosheets on alveolar cells. *Applied Surface Science*, *434*, 1274-1284. https://doi.org/https://doi.org/10.1016/j.apsusc.2017.11.217

Doak, S. H., Andreoli, C., Burgum, M. J., Chaudhry, Q., Bleeker, E. A. J., Bossa, C., Domenech, J., Drobne, D., Fessard, V., Jeliazkova, N., Longhin, E., Rundén-Pran, E., Stępnik, M., El Yamani, N., Catalán, J., & Dusinska, M. (2023). Current status and future challenges of genotoxicity OECD Test Guidelines for nanomaterials: a workshop report. *Mutagenesis*, *38*(4), 183-191. https://doi.org/10.1093/mutage/gead017

Domenech, J., Rodríguez-Garraus, A., de Cerain, A. L., Azqueta, A., & Catalán, J. (2022). Genotoxicity of Graphene-Based Materials. In *Nanomaterials* (Vol. 12): MDPI.

El-Kady, M. F., Shao, Y., & Kaner, R. B. (2016). Graphene for batteries, supercapacitors and beyond. *Nature Reviews Materials*, *1*(7), 16033. https://doi.org/10.1038/natrevmats.2016.33

El Yamani, N., Rundén-Pran, E., Collins, A. R., Longhin, E. M., Elje, E., Hoet, P., Vinković Vrček, I., Doak, S. H., Fessard, V., & Dusinska, M. (2022). The miniaturized enzyme-modified comet assay for genotoxicity testing of nanomaterials. *Front Toxicol*, *4*, 986318. https://doi.org/10.3389/ftox.2022.986318

EUON. (2022a). *Study of the EU market fornanomaterials, including substances, uses, volumes and key operators*. E. C. Agency.

EUON. (2022b). *Assessment of the potential impact of graphene, graphene oxide and other 2D materials on health, and the environment*. E. C. Agency.

Evans, S. J., Clift, M. J. D., Singh, N., Wills, J. W., Hondow, N., Wilkinson, T. S., Burgum, M. J., Brown, A. P., Jenkins, G. J., & Doak, S. H. (2019). In vitro detection of in vitro

secondary mechanisms of genotoxicity induced by engineered nanomaterials. *Particle and Fibre Toxicology*, *16*(1), 8. https://doi.org/10.1186/s12989-019-0291-7

Fadeel, B., Bussy, C., Merino, S., Vázquez, E., Flahaut, E., Mouchet, F., Evariste, L., Gauthier, L., Koivisto, A. J., Vogel, U., Martín, C., Delogu, L. G., Buerki-Thurnherr, T., Wick, P., Beloin-Saint-Pierre, D., Hischier, R., Pelin, M., Candotto Carniel, F., Tretiach, M., Cesca, F., Benfenati, F., Scaini, D., Ballerini, L., Kostarelos, K., Prato, M., & Bianco, A. (2018). Safety Assessment of Graphene-Based Materials: Focus on Human Health and the Environment. In *ACS Nano* (Vol. 12, pp. 10582-10620): American Chemical Society.

FDA. (2012). *U.S. Food & Drug Administration: Guidance for Industry: Pyrogen and Endotoxins Testing: Questions and Answers*. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-pyrogen-and-endotoxins-testing-questions-and-answers (accessed on 21 November 2022)

Geim, A. K., & Novoselov, K. S. (2007). The rise of graphene. *Nature Materials*, *6*(3), 183-191. https://doi.org/10.1038/nmat1849

Gies, V., Lopinski, G., Augustine, J., Cheung, T., Kodra, O., & Zou, S. (2019). The impact of processing on the cytotoxicity of graphene oxide. *Nanoscale Advances*, *1*(2), 817-826. https://doi.org/10.1039/c8na00178b

Guo, Z., Chakraborty, S., Monikh, F. A., Varsou, D. D., Chetwynd, A. J., Afantitis, A., Lynch, I., & Zhang, P. (2021). Surface Functionalization of Graphene-Based Materials: Biological Behavior, Toxicology, and Safe-By-Design Aspects. *Advanced Biology*, *5*(9), 2100637-2100637. https://doi.org/10.1002/adbi.202100637

Huang, X., Yin, Z., Wu, S., Qi, X., He, Q., Zhang, Q., Yan, Q., Boey, F., & Zhang, H. (2011). Graphene-based materials: Synthesis, characterization, properties, and applications. In *Small* (Vol. 7, pp. 1876-1902).

Jarosz, A., Skoda, M., Dudek, I., & Szukiewicz, D. (2016). Oxidative Stress and Mitochondrial Activation as the Main Mechanisms Underlying Graphene Toxicity against Human Cancer Cells. *Oxid Med Cell Longev*, *2016*, 5851035. https://doi.org/10.1155/2016/5851035

Kermanizadeh, A., Chauché, C., Brown, D. M., Loft, S., & Møller, P. (2015). The role of intracellular redox imbalance in nanomaterial induced cellular damage and genotoxicity: a review. *Environ Mol Mutagen*, *56*(2), 111-124. https://doi.org/10.1002/em.21926

Könczöl, M., Ebeling, S., Goldenberg, E., Treude, F., Gminski, R., Gieré, R., Grobéty, B., Rothen-Rutishauser, B., Merfort, I., & Mersch-Sundermann, V. (2011). Cytotoxicity and genotoxicity of size-fractionated iron oxide (magnetite) in A549 human lung epithelial cells: role of ROS, JNK, and NF-κB. *Chem Res Toxicol*, *24*(9), 1460-1475. https://doi.org/10.1021/tx200051s

Lee, C., Wei, X., Kysar, J. W., & Hone, J. (2008). Measurement of the Elastic Properties and Intrinsic Strength of Monolayer Graphene. *Science*, *321*(5887), 385-388. https://doi.org/doi:10.1126/science.1157996

Lee, J. H., Han, J. H., Kim, J. H., Kim, B., Bello, D., Kim, J. K., Lee, G. H., Sohn, E. K., Lee, K., Ahn, K., Faustman, E. M., & Yu, I. J. (2016). Exposure monitoring of graphene nanoplatelets manufacturing workplaces. *Inhalation Toxicology*, *28*(6), 281-291. https://doi.org/10.3109/08958378.2016.1163442

Lee, Y.-S., Sung, J.-H., Song, K.-S., Kim, J.-K., Choi, B.-S., Yu, I.-J., & Park, J.-D. (2019). Derivation of occupational exposure limits for multi-walled carbon nanotubes and graphene

using subchronic inhalation toxicity data and a multi-path particle dosimetry model. *Toxicology Research*, *8*(4), 580-586. https://doi.org/10.1039/c9tx00026g

Li, R., Guiney, L. M., Chang, C. H., Mansukhani, N. D., Ji, Z., Wang, X., Liao, Y. P., Jiang, W., Sun, B., Hersam, M. C., Nel, A. E., & Xia, T. (2018). Surface Oxidation of Graphene Oxide Determines Membrane Damage, Lipid Peroxidation, and Cytotoxicity in Macrophages in a Pulmonary Toxicity Model. *ACS Nano*, *12*(2), 1390-1402. https://doi.org/10.1021/acsnano.7b07737

Liao, K.-H., Lin, Y.-S., Macosko, C. W., & Haynes, C. L. (2011). Cytotoxicity of Graphene Oxide and Graphene in Human Erythrocytes and Skin Fibroblasts. *ACS Applied Materials & Interfaces*, *3*(7), 2607-2615. https://doi.org/10.1021/am200428v

Lin, W., Huang, Y. W., Zhou, X. D., & Ma, Y. (2006). In vitro toxicity of silica nanoparticles in human lung cancer cells. *Toxicol Appl Pharmacol*, *217*(3), 252-259. https://doi.org/10.1016/j.taap.2006.10.004

Lugrin, J., Rosenblatt-Velin, N., Parapanov, R., & Liaudet, L. (2014). The role of oxidative stress during inflammatory processes. *Biol Chem*, *395*(2), 203-230. https://doi.org/10.1515/hsz-2013-0241

Luo, B., Liu, S., & Zhi, L. (2012). Chemical Approaches toward Graphene-Based Nanomaterials and their Applications in Energy-Related Areas. *Small*, *8*(5), 630-646. https://doi.org/https://doi.org/10.1002/smll.201101396

Magne, T. M., de Oliveira Vieira, T., Alencar, L. M. R., Junior, F. F. M., Gemini-Piperni, S., Carneiro, S. V., Fechine, L. M. U. D., Freire, R. M., Golokhvast, K., Metrangolo, P., Fechine, P. B. A., & Santos-Oliveira, R. (2022). Graphene and its derivatives: understanding the main chemical and medicinal chemistry roles for biomedical applications. *Journal of Nanostructure in Chemistry*, *12*(5), 693-727. https://doi.org/10.1007/s40097-021-00444-3

Majeed, W., Bourdo, S., Petibone, D. M., Saini, V., Vang, K. B., Nima, Z. A., Alghazali, K. M., Darrigues, E., Ghosh, A., Watanabe, F., Casciano, D., Ali, S. F., & Biris, A. S. (2017). The role of surface chemistry in the cytotoxicity profile of graphene. *Journal of Applied Toxicology*, *37*(4), 462-470. https://doi.org/10.1002/jat.3379

Mittal, S., Kumar, V., Dhiman, N., Chauhan, L. K. S., Pasricha, R., & Pandey, A. K. (2016). Physico-chemical properties based differential toxicity of graphene oxide/reduced graphene oxide in human lung cells mediated through oxidative stress. *Scientific Reports*, *6*(1), 39548-39548. https://doi.org/10.1038/srep39548

OECD. (2022). *Study Report and Preliminary Guidance on the Adaptation of the In Vitro micronucleus assay (OECD TG 487) for Testing of Manufactured Nanomaterials*.

OECD. (2023). *Test No. 487: In Vitro Mammalian Cell Micronucleus Test*. https://doi.org/doi:https://doi.org/10.1787/9789264264861-en

Olabi, A. G., Abdelkareem, M. A., Wilberforce, T., & Sayed, E. T. (2021). Application of graphene in energy storage device – A review. *Renewable and Sustainable Energy Reviews*, *135*, 110026. https://doi.org/https://doi.org/10.1016/j.rser.2020.110026

Ou, L., Lv, X., Wu, Z., Xia, W., Huang, Y., Chen, L., Sun, W., Qi, Y., Yang, M., & Qi, L. (2021). Oxygen content-related DNA damage of graphene oxide on human retinal pigment epithelium cells. *Journal of Materials Science: Materials in Medicine*, *32*(2). https://doi.org/10.1007/s10856-021-06491-0

Park, M. V. D. Z., Bleeker, E. A. J., Brand, W., Cassee, F. R., van Elk, M., Gosens, I., de Jong, W. H., Meesters, J. A. J., Peijnenburg, W. J. G. M., Quik, J. T. K., Vandebriel, R. J., & Sips, A. J. A.

M. (2017). Considerations for Safe Innovation: The Case of Graphene. *ACS Nano*, *11*(10), 9574-9593. https://doi.org/10.1021/acsnano.7b04120

Pei, S., & Cheng, H. M. (2012). The reduction of graphene oxide. *Carbon*, *50*(9), 3210-3228. https://doi.org/10.1016/j.carbon.2011.11.010

Pelin, M., Fusco, L., León, V., Martín, C., Criado, A., Sosa, S., Vázquez, E., Tubaro, A., & Prato, M. (2017). Differential cytotoxic effects of graphene and graphene oxide on skin keratinocytes. *Scientific Reports*, *7*(1), 40572. https://doi.org/10.1038/srep40572

Pelin, M., Fusco, L., Martín, C., Sosa, S., Frontiñán-Rubio, J., González-Domínguez, J. M., Durán-Prado, M., Vázquez, E., Prato, M., & Tubaro, A. (2018). Graphene and graphene oxide induce ROS production in human HaCaT skin keratinocytes: the role of xanthine oxidase and NADH dehydrogenase [10.1039/C8NR02933D]. *Nanoscale*, *10*(25), 11820-11830. https://doi.org/10.1039/C8NR02933D

Pelin, M., Passerino, C., Rodríguez-Garraus, A., Carlin, M., Sosa, S., Suhonen, S., Vales, G., Alonso, B., Zurutuza, A., Catalán, J., & Tubaro, A. (2023). Role of Chemical Reduction and Formulation of Graphene Oxide on Its Cytotoxicity towards Human Epithelial Bronchial Cells. *Nanomaterials*, *13*(15), 2189. https://www.mdpi.com/2079-4991/13/15/2189

Pelin, M., Sosa, S., Prato, M., & Tubaro, A. (2018). Occupational exposure to graphene based nanomaterials: risk assessment. *Nanoscale*, *10*(34), 15894-15903. https://doi.org/10.1039/c8nr04950e

Ray, S. C. (2015). Application and Uses of Graphene Oxide and Reduced Graphene Oxide. In *Applications of Graphene and Graphene-Oxide Based Nanomaterials* (pp. 39-55). Elsevier. https://doi.org/10.1016/B978-0-323-37521-4.00002-9

Razaq, A., Bibi, F., Zheng, X., Papadakis, R., Jafri, S. H. M., & Li, H. (2022). Review on Graphene-, Graphene Oxide-, Reduced Graphene Oxide-Based Flexible Composites: From Fabrication to Applications. In *Materials* (Vol. 15): MDPI.

Reina, G., González-Domínguez, J. M., Criado, A., Vázquez, E., Bianco, A., & Prato, M. (2017). Promises, facts and challenges for graphene in biomedical applications. In *Chemical Society Reviews* (Vol. 46, pp. 4400-4416): Royal Society of Chemistry.

Rodríguez-Garraus, A., Passerino, C., Vales, G., Carlin, M., Suhonen, S., Tubaro, A., Gómez, J., Pelin, M., & Catalán, J. (2023). Impact of physico-chemical properties on the toxicological potential of reduced graphene oxide in human bronchial epithelial cells. *Nanotoxicology*, *(in press)*.

Tarelho, J. P. G., Soares dos Santos, M. P., Ferreira, J. A. F., Ramos, A., Kopyl, S., Kim, S. O., Hong, S., & Kholkin, A. (2018). Graphene-based materials and structures for energy harvesting with fluids – A review. *Materials Today*, *21*(10), 1019-1041. https://doi.org/https://doi.org/10.1016/j.mattod.2018.06.004

Tombolini, F., Boccuni, F., Ferrante, R., Natale, C., Marasco, L., Mantero, E., Del Rio Castillo, A. E., Leoncino, L., Pellegrini, V., Sabella, S., & Iavicoli, S. (2021). An integrated and multi-technique approach to characterize airborne graphene flakes in the workplace during production phases [10.1039/D0NR07114E]. *Nanoscale*, *13*(6), 3841-3852. https://doi.org/10.1039/D0NR07114E

Ursini, C. L., Fresegna, A. M., Ciervo, A., Maiello, R., Del Frate, V., Folesani, G., Galetti, M., Poli, D., Buresti, G., Di Cristo, L., Sabella, S., Iavicoli, S., & Cavallo, D. (2021). Occupational exposure to graphene and silica nanoparticles. Part II: pilot study to identify a panel of sensitive biomarkers of genotoxic, oxidative and inflammatory effects on suitable

biological matrices. *Nanotoxicology*, *15*(2), 223-237. https://doi.org/10.1080/17435390.2020.1850903

Wick, P., Louw-Gaume, A. E., Kucki, M., Krug, H. F., Kostarelos, K., Fadeel, B., Dawson, K. A., Salvati, A., Vázquez, E., Ballerini, L., Tretiach, M., Benfenati, F., Flahaut, E., Gauthier, L., Prato, M., & Bianco, A. (2014). Classification framework for graphene-based materials. In *Angewandte Chemie - International Edition* (Vol. 53, pp. 7714-7718): Wiley-VCH Verlag.

Xiang, Y., Xin, L., Hu, J., Li, C., Qi, J., Hou, Y., & Wei, X. (2021). Advances in the Applications of Graphene-Based Nanocomposites in Clean Energy Materials. *Crystals*, *11*(1), 47. https://www.mdpi.com/2073-4352/11/1/47

Yang, H., Liu, C., Yang, D., Zhang, H., & Xi, Z. (2009). Comparative study of cytotoxicity, oxidative stress and genotoxicity induced by four typical nanomaterials: the role of particle size, shape and composition. *J Appl Toxicol*, *29*(1), 69-78. https://doi.org/10.1002/jat.1385

Yang, Z., Pan, Y., Chen, T., Li, L., Zou, W., Liu, D., Xue, D., Wang, X., & Lin, G. (2020). Cytotoxicity and Immune Dysfunction of Dendritic Cells Caused by Graphene Oxide. *Front Pharmacol*, *11*, 1206. https://doi.org/10.3389/fphar.2020.01206

Annex I. Description of the different tasks performed within the GrapHazar project.

Task	Leader	Distribution of work
T1: Characterization of graphene-based materials (GBMs)	UniTs	UniTs: performing several physico-chemical characterization analyses of selected GBMs and setting up a method for assessing endotoxin content FIOH: contributing to the assessment of endotoxins
T2: Adjustment of test protocols for assessing GBMs	FIOH	FIOH: selection of genotoxicity assays appropriate for assessing GBMs and setting up of the flow-cytometry based micronucleus assay. UniTs: application of standardized procedures to obtain dispersions of powder GBMs and adoption of methods for cytotoxicity analysis not giving interferences with GBMs
T3: In vitro toxicity assessment of primary effects of GBMs	FIOH	FIOH: assessment of cytotoxicity and genotoxicity of GBMs UniTs: assessment of cytotoxicity, ROS production and cytokine release induced by GBMsy
T4: In vitro toxicity assessment of secondary effects of GBMs	FIOH	FIOH: Establishing a co-culture system for assessing secondary genotoxicity UniTs: Set up and adoption of a co-culture system for assessing secondary inflammatory effects
T5: Establishing correlations between physico-chemical properties of GBMs and their in vitro toxicity	UniTs	UniTs: collecting data and leading the work related to the case-study 1 FIOH: collecting data and leading the work related to the case-study 2
T6: Correlations between in vitro toxicity data and human data from workers exposed to GBMs	FIOH	FIOH: collecting information from occupational studies in the Graphene Flagship and editing a factsheet on best practices for safe graphene work UniTs: contributing to the factsheet
T7: Dissemination of the results	FIOH	Both partners disseminated the results of the project in different social media and scientific congresses and have collaborated together for publishing the results in scientific journals

This publication is the final report of the research project “Hazard characterization of graphene-based nanomaterials in energy production and storage (GrapHazard)”, funded by the Finnish Work Environmental Fund, the Italian National Institute for Insurance against Accidents at Work (INAIL), the Finnish Institute of Occupational Health, and the University of Trieste in the frame of the SAF€RA 2020 program. The report summarizes the findings of the toxicological assessment of different types of graphene-based materials by using an in vitro lung system to investigate the involved mechanisms of action and how different key physicochemical properties of the materials can modulate the toxic response. In addition, recommendations for a safe use of graphene-based materials in occupational settings are provided.

Työsuojelurahasto
Arbetarskyddsfonden
The Finnish Work Environment Fund

INAIL
ISTITUTO NAZIONALE PER L'ASSICURAZIONE CONTRO GLI INFORTUNI SUL LAVORO

Työterveyslaitos
Arbetshälsoinstitutet
Finnish Institute of Occupational Health

PL 40, 00032 Työterveyslaitos

www.ttl.fi

ISBN XXXXX (PDF)