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Short communication

An *in vitro* comparison of the toxicological profiles of ground tire particles (TP) and actual tire and road wear particles (TRWP) emissions

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ABSTRACT

There is currently limited data on the potential effects of tire and road wear particles (TRWP) on human health. TRWP include tire fragments, but also road wear materials, dust, adsorbed gaseous pollutants and different types of inclusions that could affect their hazard profiles. Due to their availability and lower complexity, ground tire particles (TP) are often used in toxicological studies. However, this makes it difficult to draw firm conclusions about the potential hazard of actual TRWP. Here, we compared the *in vitro* toxicological profile of ground TP and actual TRWP emissions of similar size collected from road traffic. For this purpose, TP and TRWP were separately incubated with alveolar macrophages for 24 h, and the cellular response was evaluated in terms of cytotoxicity, proinflammatory response and oxidative stress. Both TP and TRWP induced neither significant cytotoxicity nor oxidative stress, but triggered a concentration-dependent proinflammatory response, as evidenced by increased TNF- α production. The level of TNF- α production was slightly higher with TRWP than with TP, independent of the particle dose. All in all, the pulmonary toxicity of TRWP could be due primarily to the tire tread inclusions and only marginally to other particle components (*i.e.* road wear materials, dust ...). Although these preliminary results need to be confirmed by further analysis, they could be useful for tire manufacturers in the production of safer-by-design tires.

1. Introduction

Air pollution is a major public health problem. Among air pollutants, particulate matter (PM) has been classified as a Group I human carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 2013; Loomis et al., 2013), and a clear association has been established between exposure to PM and adverse health effects such as respiratory and cardiovascular diseases and cancers (Cazzolla Gatti, 2021; Charloux et al., 1997; Hamra et al., 2014; Kurt et al., 2016; Mayoralas-Alises and Diaz-Lobato, 2012; Nishida and Yatera, 2022; Raaschou-Nielsen et al., 2013; Tseng et al., 2019; Wong, 2014). In fact, the European Environment Agency (EEA) reported that more than 250,000 premature deaths were attributable to fine PM emissions in 2021 (European Environment Agency, 2023).

Pollution from road traffic is a major contributor to PM emissions, especially in urban areas. PM from road traffic originates from either exhaust emissions (EE, from the tailpipe) or non-exhaust emissions

(NEE) (Lewis et al., 2019). A significant proportion of particulate NEE results from mechanical abrasion of brakes and tires, erosion of road surfaces, and resuspension of road dust into the atmosphere by passing traffic (Fussell et al., 2022; Harrison et al., 2021; Piscitello et al., 2021). While EE has been extensively studied and significantly reduced due to the introduction of stricter emission standards, the unregulated particulate NEE fraction has prevailed and now exceeds EE in many countries (ADEME, 2022; Amato et al., 2014; Baensch-Baltruschat et al., 2020; Fussell et al., 2022; Harrison et al., 2021; Lewis et al., 2019; Piscitello et al., 2021). Particulate NEE, which is a significant source of PM10 and PM2.5 in urban environments, has not received as much attention and there is currently no legislation to specifically reduce NEE particles. Consequently, very few data are available on the potential effects of particulate NEE on human health (Bouredji et al., 2023; Forest and Pourchez, 2023) and it is therefore of paramount importance to assess their toxicological effects.

Tire and road wear particles (TRWP) make up a large proportion of

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NEE particles. They are generated by friction between the tire tread and the road surface, resulting in tire abrasion and/or evaporation and subsequent condensation of the volatile compounds. In most cases, the coarse TRWP particles emitted consist of tire wear fragments embedded in a matrix of road surface compounds and environmental pollutants, including worn materials and deposited dust (Harrison et al., 2021; Lewis et al., 2019). Therefore, TRWP emission and composition depend on many parameters such as chemical composition of tires, type of road surface, driving style, climate, etc. It also follows that the *in vitro* or *in vivo* effects triggered by TRWP can be significantly influenced by their composition and ultimately by their conditions of formation.

In the literature, mainly three different sources of tire particles have been used for toxicological studies: particles obtained by grinding of the tire tread, particles produced using a road simulator, or particles collected on the road. In the first method, the particles are usually abraded from tire treads that have never been used and therefore do not contain road wear materials and other types of contaminants; they are usually referred to as tire particles (TP). Particles generated by tire abrasion on a road simulator contain some of the road wear but not all of the contaminants normally deposited on real roads, and environmental pollutants; they are referred to as tire wear particles (TWP). Finally, the particles collected on the road are a mixture of tire particles, road dust and other car emissions and are therefore most representative of real-world emissions (Piscitello et al., 2021). As the composition of tire particles depends on the way they are produced, comparisons between studies from the literature are difficult, making it difficult to draw firm conclusions about the potential toxicity of TRWP.

To address this gap, we compared the *in vitro* toxicological profile of TP obtained by grinding to that of on-road collected TRWP. We tried to answer the question: "What are the particle constituents responsible for the pulmonary toxicity of TRWP?". The comparison of TP and TRWP, which have a similar size but differ in their formation process and thus in their composition, will allow a better understanding of the toxicity of TRWP in relation to their extragenic inclusions. The research will also provide a solid basis for assessing the materiality of toxicity results based solely on TP samples.

2. Materials and methods

2.1. Generation of TP and TRWP samples

TP were obtained by cryogenic grinding of a tire (CryoMill by Resch, Verder International BV, Netherlands). 10 grinding cycles were performed in a zirconium oxide container containing 3 g of tire particles (≈ 1 mm). Each grinding cycle lasted 2 min at 30 Hz under liquid nitrogen. Size fractionation was then performed and the particles were collected on 5 μm and 15 μm sieves. The sieving process was carried out in 10 min cycles at a rate of 2 mm/G in the presence of ethanol.

TRWP on the road were collected using a fully instrumented Clio 3 (1.6i 16 V, 111 hp) equipped with a high volume air sampler (HVS, HiVol Plus from Tisch). The vehicle was fitted with standard summer tires, and the same type of tire was used to produce the TP powders. Sampling took place in the Nantes agglomeration. Briefly, the air intake for TRWP collection was placed behind the front wheels on the passenger side (De Oliveira et al., 2024). It was positioned and oriented according to Rajaratnam and Walker (Rajaratnam and Walker, 2019) and Noh et al. (Noh et al., 2021) to capture particles originating from both the tire-road interface and the resuspension caused by the tires. The HVS suction velocity at the sample inlet (3.5 m/s) was between the average airflow velocity at the rear of the wheels in urban areas (about 1.6 m/s) and on highways (up to 6.1 m/s). The HVS unit was equipped with a HiVol cascade impactor from Tisch® Series-230 consisting of five stacked impaction plates with mean cut-off diameters ranging from 0.69 μm to > 10 μm (specifically 10.2, 4.2, 2.1, 1.3 and 0.69 μm) for a nominal airflow rate of 566 L/min. A slotted cellulose collection substrate was located on each plate to collect the particles and prevent them

from bouncing off on impact. A terminal collection substrate for the total suspended particles was used to collect all particles < 0.39 μm (<https://tisch-env.com/impactors/hivol-cascade-impactors/>). Once collected, TRWP were retrieved from the collection substrates by successive ultrapure water washing and sonication steps followed by slow evaporation (at 50 °C) until a solution with a final TRWP concentration of > 15 mg/mL was obtained. The recovery was determined by drying and then weighing the collection substrates before and after TRWP separation and was above 85 %. In an effort to maintain consistency between TP and on-road TRWP samples, only the toxicological profiles of those TRWP collected on impaction plates with mean cut-off diameters of 4.2 μm and > 10 μm were used here for comparison.

2.2. Physicochemical characterization

The morphology of the particles was examined using scanning electron microscopy (SEM). The SEM images were taken with a Zeiss ULTRA-Plus microscope equipped with a field emission gun and an SE detector in the objective. The particle size distribution was determined using a laser diffraction size analyzer (Mastersizer 3000, Malvern Instruments Ltd, Worcestershire, UK). In addition, the specific surface area of the particles was measured using the Brunauer-Emmett-Teller (BET) method (analysis of the adsorption of krypton, performed with a Micrometrics TRI-STAR 3000 analyzer). Finally, the chemical composition of the particles after mineralization of the samples was analyzed using inductively coupled plasma mass spectrometry (ICP-MS) with an Agilent 7800 Inductively Coupled Plasma Spectroscopy.

2.3. *In vitro* toxicity assessment

2.3.1. Cell culture

The RAW 264.7 cell line was derived from mouse macrophages transformed by Abelson murine leukemia virus and was provided by the ATCC Cell Biology Collection (Promochem, LGC, Molsheim, France). Cells were cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10 % fetal calf serum and 1 % penicillin–streptomycin at 37 °C in an atmosphere humidified with 5 % carbon dioxide. The cells were seeded in 96-well plates (100,000 cells in 50 μL medium per well) and allowed to attach overnight. The particles were diluted in the cell culture medium to achieve the following final concentrations: 15, 30, 60 and 120 $\mu\text{g}/\text{mL}$. After a 24 h incubation with the particles, the cell response was evaluated in terms of cytotoxicity (assessed by the release of lactate dehydrogenase), proinflammatory response (measurement of TNF- α production) and induction of oxidative stress (assessed by the production of reactive oxygen species, ROS).

2.3.2. Cytotoxicity assessment

Cytotoxicity was assessed by analyzing cell membrane integrity by measuring the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) in cell culture supernatant using the CytoTox-96™ Homogeneous Membrane Integrity Assay (Promega, Charbonnières-les-Bains, France) according to the manufacturer's instructions. The optical density of the samples was determined using a microplate reader set at 450 nm (Multiskan RC; ThermoFisher, Helsinki, Finland). Three independent experiments were performed in quadruplicate, and the activity of the released LDH was compared with the activity of negative control cells (incubated without particles). The maximum cellular LDH released after lysis of the cells served as a positive control.

2.3.3. Proinflammatory response

After 24 h of contact between particles and cells, the production of TNF- α , a proinflammatory cytokine, in cell culture medium was determined using a commercial ELISA kit (Quantikine® Mouse TNF- α Immunoassay; R&D Systems, Lille, France) according to the manufacturer's instructions. The optical density of each sample was determined using a microplate reader set at 450 nm (Multiskan RC;

Thermolabsystems, Helsinki, Finland). A standard curve was established and the results were expressed in picograms of TNF- α per milliliter of supernatant. Three independent experiments were performed in quadruplicate, and TNF- α production was compared with that of control cells (incubated without particles).

2.3.4. Oxidative stress

The OxiSelect™ ROS Assay Kit (Euromedex, Mundolsheim, France) can be used to assess a wide range of ROS activities. The assay utilizes the conversion of a non-fluorescent substrate, 2,7'-dichlorodihydrofluorescein diacetate, which can easily diffuse through cell membranes and be converted to a fluorogenic molecule 2',7'-dichlorodihydrofluorescein (DCF) in the presence of ROS, with the amount of fluorescence directly related to the ROS content. Fluorescence was measured using a Fluoroskan Ascent fluorometer (Ex: 480 nm, Em: 530 nm, Thermolabsystems) after a 90 min and a 24 h incubation of the cells with the particles. Incubation of the cells with H₂O₂ (1 mM) was used as a positive control. Three independent experiments were performed in quadruplicate, and ROS formation was compared with that of the negative control (cells incubated without particles).

2.4. Statistical analyses

Analyses and graphs were performed using Prism software (Graph-Pad, San Diego, CA). Significance was established with two-way ANOVA tests and Tukey post-tests ($***p < 0.001$ and $*p < 0.05$). Each data point represents the mean of at least three independent experiments and is presented with the arithmetic standard error of the mean (\pm SEM).

3. Results and discussion

3.1. Physicochemical characterization of the particles

Fig. 1 illustrates the morphology of the particles. The TP were characterized by small particles that adhere to larger ones (Fig. 1A). Ground TP have a relatively similar appearance to the supermicronic TRWP collected on-road. The latter generally have an elongated morphology and a relatively irregular or granular texture (Fig. 1B). This type of morphology and texture generally reflects the amount of inclusions they contain and the intensity of the mixing processes they have undergone at the tire-road interface. However, due to the multiple processes responsible for their formation, these characteristics vary greatly depending on the route driven, the driving style or the age (and therefore the degree of degradation) of the particles. It should be noted that the collected TRWP might have evolved during their extraction

from the collection substrate. The labile TRWP fractions were nevertheless preserved in the solution used for the toxicity tests, as was the refractory fraction (e.g. tire fragments). In fact, once TRWP have been introduced into ecosystems or organisms, they are also susceptible to leaching and slow dissolution mechanisms.

The analysis of the size distribution of the TP revealed an average size of 22 μ m, with a D₁₀ of 10 μ m (average size of the smallest particles) and a D₉₀ of 37 μ m (average size of the largest particles). The size of TRWP varies greatly depending on the route, traffic conditions or driving style. Size distribution data obtained from microscopic observations of the HVS collection substrate generally show bi- or trimodal distributions with stronger contributions from the $< 0.7 \mu$ m and $> 2 \mu$ m fractions. These two fractions were also observed by Beji et al. (Beji et al., 2021), and the authors emphasized that the coarse TRWP fraction exceeds 20 μ m. In our case, this coarse TRWP fraction is 25–30 μ m, while some TRWP exceed 80 μ m. This indicates that the initial properties (size and shape) of the particles being compared are relatively similar and that much of the difference in toxicity is primarily due to the contrast in chemical composition.

The chemical analysis of TP revealed, in addition to C (which accounts for 60–80 % of the tire tread mass), a significant amount of Si (in the order of 3–8 %, as silicon is used as a filler). In addition, significant amounts (in the order of 1 %) of S (sulfur is used in the vulcanization process), Zn (zinc is used as a catalyst in tire treatment processes) and Fe were found. Other trace elements (with contents between 1 % and 0.1 % or less) were quantified, such as sodium (Na), calcium (Ca) and aluminum (Al). Lower and more variable C contents were measured in the coarse TRWP: typically between 30 % and 60 %. Due to the variety of inclusions, the average Si, Fe and Al contents increased to 4–10 %, 1–4 % and 1–3 % respectively. Locally, higher contents of Ca, Mg, Na and K were also found (up to a few percent). Finally, using TP as a reference, other elements were detected in traces, such as Cu, Cr, Sn, Ni, Pt and Ti, depending on the route traveled.

It is important to point out that, in addition to the mineral and metallic components of TP and TRWP, tires contain a wide range of soluble organic compounds whose toxicity is now generally recognized. Similarly, emitted particles can undergo degradation and ageing processes leading to the formation of hazardous products, an example is the 6PPD which reacts with ozone to produce toxic 6PPD quinone (Tian et al., 2021; Xu et al., 2022). Our tests therefore evaluate the toxicity of TP containing all these organic compounds and compare it with that of coarse TRWP (in which tire inclusions are only one of their components), which contain a smaller amount of the same pollutants, but to which are added the substances originating from road wear materials, dust and various types of inclusions.

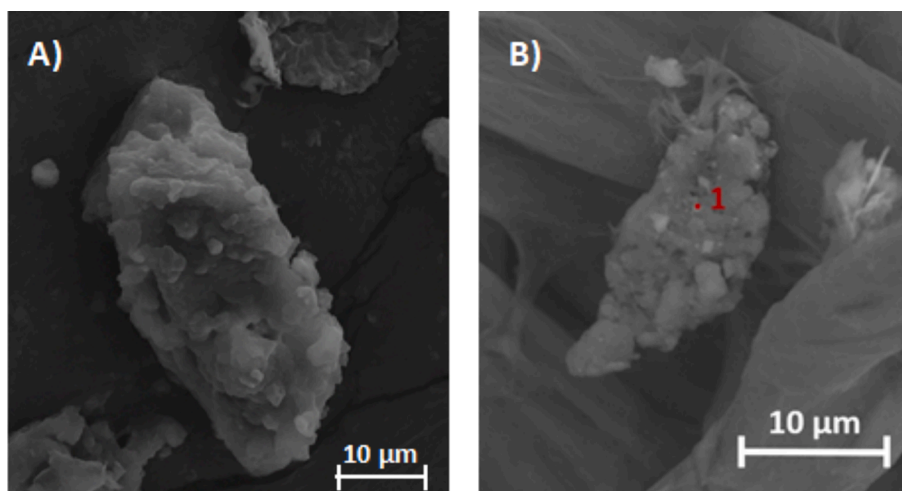


Fig. 1. Morphology of TP (A) and coarse TRWP (B) observed by scanning electron microscopy.

3.2. *In vitro* cellular response

For *in vitro* toxicity assessment, TP or coarse TRWP ($4.2 - >10 \mu\text{m}$) were incubated for 24 h with cells of the RAW264.7 cell line (alveolar macrophages representing the first line of defense against inhaled pollutants). Results are shown in Fig. 2. TP and TRWP showed a similar toxicological profile. They induced neither a significant cytotoxicity nor an oxidative stress but triggered a concentration-dependent proinflammatory response. The amount of TNF- α produced was slightly higher with TRWP than with TP, independent of the particle dose.

These findings are consistent with studies from the literature reporting that TP can induce a proinflammatory response both *in vitro* (Jiang et al., 2022; Liu et al., 2022; Poma et al., 2019) and *in vivo* (Li et al., 2022; Mantecca et al., 2010; Mantecca et al., 2009). Similarly, coarse TRWP were found to enhance the production of proinflammatory cytokines, in agreement with previous studies (Gustafsson et al., 2008; Karlsson et al., 2011, 2006; Lindbom et al., 2007, 2006).

The hazard observed for TP reflects the intrinsic toxicity of the matrix (*i.e.* the tire tread material). Coarse TRWP, on the other hand, include road wear material, dust, adsorbed gaseous pollutants and various types of inclusions. Since TP represent a variable and locally small fraction of TRWP, the toxicity of TRWP could also stem from all these additional components, including brake wear components. People living near roads, especially those exposed to the urban pollution cloud, would therefore inhale a wide range of TRWP of variable toxicity. A strong point of this research lies in the evaluation of the toxicity of real TRWP, rather than that of uniform TRWP simulated on chassis dynamometers: the latter often overlook a large and essential part of the complexity of emissions generated by tire-road contact. Interestingly, however, despite the variety of real TRWP and the profound

compositional differences between TP and TRWP, their toxicity profiles were very similar. This points to the tire contribution, although sometimes in the minority, as the major factor in the toxicity of TRWP. The other components of TRWP (in the mass proportion in which they are present) would play a minor role, although not negligible, as suggested by the higher amount of TNF- α induced by TRWP. Furthermore, assuming that TRWP represent only a limited fraction of the collected emissions (Beji et al., 2021; Wik and Dave, 2009), our data suggest that the toxicological profile and the proinflammatory response of the rear-of-wheel particle emissions (not just TRWP) may also be significantly shaped by those of their tire components.

In conclusion, although preliminary, our results suggest that tire composition has a significant impact on the hazard of TRWP and total particle emissions at the rear of the wheel. If confirmed, these results could help tire manufacturers to produce safer-by-design tires. From a health and environmental point of view, our results also raise the question of the toxicity of aged emissions, *e.g.* TRWP deposited on/around roads and eventually released into the atmosphere and other parts of the ecosphere. Furthermore, the information obtained needs to be supplemented by comparative toxicity data for fine ($<1 \mu\text{m}$) or even ultrafine ($<0.1 \mu\text{m}$) TRWP fractions or particulate emissions behind the wheel. Preliminary microscopic observations in our laboratory suggest that fine and ultrafine TRWP, formed by thermo-mechanical decomposition of the tire tread under severe mechanical conditions (*e.g.* during hard braking or sharp cornering), may consist of less embedded TRWP, resulting in toxicities even closer to those of TP of comparable size. Finally, in order to make an accurate risk assessment this study on the hazard of tire particles should be complemented by exposure studies.

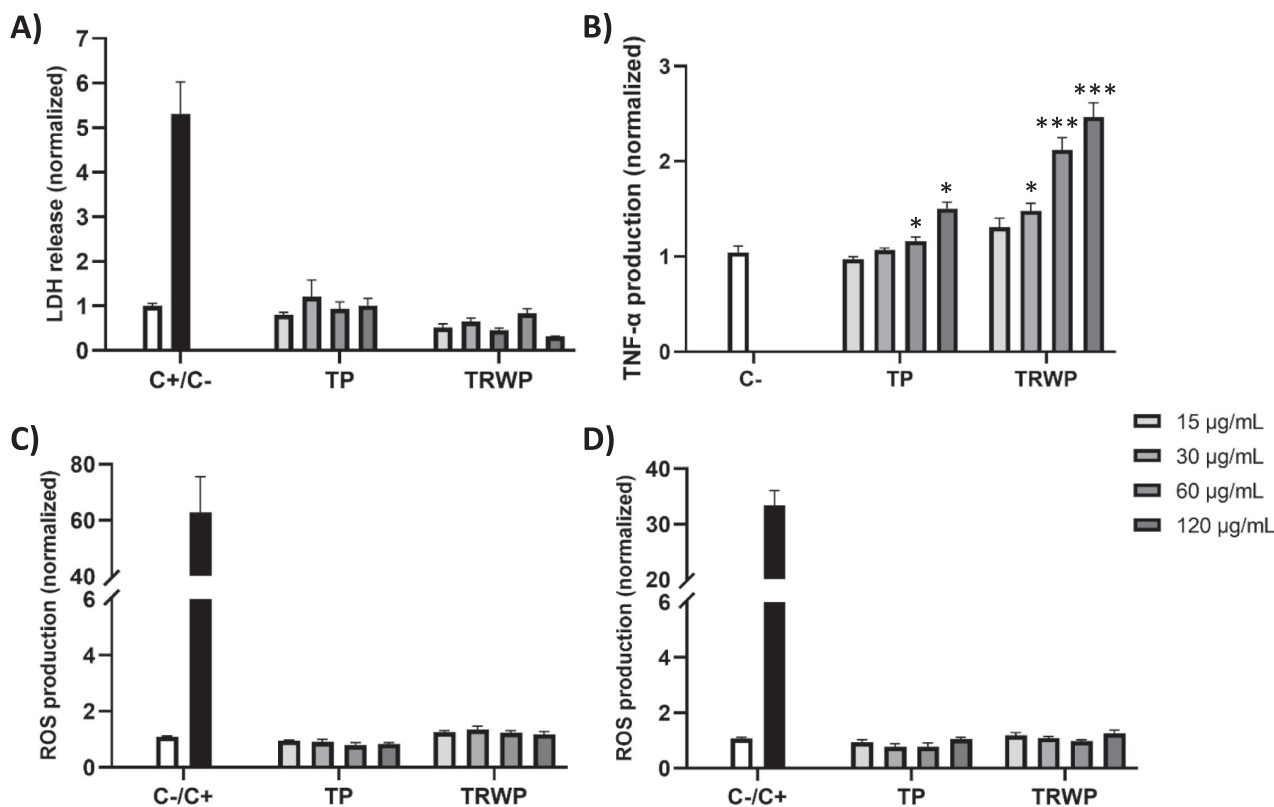


Fig. 2. *In vitro* toxicological profiles of TP and coarse TRWP: relative cytotoxicity assessed by LDH release after a 24 h cells/particles contact (A), relative TNF- α production after a 24 h cells/particles contact (B) and ROS production after a 90 min (C) or 24 h cells/particles contact (D). All results are normalized (*i.e.* expressed relative to control, unexposed, cells) and are expressed as means of three independent experiments performed in quadruplicate. Standard errors of the mean are also indicated. * $p < 0.05$, *** $p < 0.001$ as determined with two-way ANOVA tests and Tukey post-tests. C-: negative control (cells incubated without particles), C+: positive control (for LDH assay: lysed cells, for ROS: cells incubated with 1 mM H_2O_2).

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CRedit authorship contribution statement

Abderrahmane Bouredji: Writing – review & editing, Methodology, Investigation. **Bogdan Muresan:** Writing – review & editing, Supervision, Resources, Conceptualization. **Xuan-Trinh Truong:** Writing – review & editing, Investigation. **Laurence Lumière:** Writing – review & editing, Investigation. **Jérémy Pourchez:** Writing – review & editing, Supervision, Conceptualization. **Valérie Forest:** Writing – original draft, Visualization, Supervision, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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