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Increased telomere length and mtDNA copy number induced by multi-walled carbon nanotube exposure in the workplace

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Abstract: Carbon nanotubes (CNTs) – except MWCNT-7 - have been classified as Group 3 ["*Not classifiable as to its carcinogenicity to humans*"] by the IARC. Despite considerable mechanistic evidence *in vitro/ in vivo*, the classification highlights a general lack of data, especially among humans. In our previous study, we reported epigenetic changes in the MWCNT exposed workers. Here, we evaluated whether MWCNT can also cause alterations in aging related features including relative telomere length (TL) and/or mitochondrial copy number (mtDNAcn). Relative TL and mtDNAcn were measured on extracted DNA from peripheral blood from MWCNT exposed workers (*N* = 24) and non-exposed controls (*N* = 43) using a qPCR method. A higher mtDNAcn and longer TL were observed in MWCNT exposed workers when compared to controls. Independent of age, sex, smoking behavior, alcohol consumption and BMI, MWCNT-exposure was associated with an 18.30 % increase in blood TL (95% CI: 7.15 to 30.62 %; p = 0.001) and 35.21 % increase in mtDNAcn (95% CI: 19.12 to 53.46 %). Our results suggest that exposure to MWCNT can induce an increase in the mtDNAcn and TL; however, the mechanistic basis or consequence of such change requires further experimental studies.

Keywords: nanotoxicology; carbon nanotubes; occupational exposure; telomere length; mitochondrial DNA

1. Introduction

Carbon nanotubes (CNTs) with its specific electrical and thermal properties, and unique mechanical properties, have a great potential for commercialization. On the other hand, there are concerns about its effect on worker/consumer health from exposure during manufacturing/handling and the use of consumer products. Studies thus far have established the toxicity and potential carcinogenicity of several forms of CNTs, in *vitro* and in rodent models. Since no human cancer data are available, the International Agency for Research on Cancer (IARC) focused on these results assessing the mechanism of toxicity and carcinogenicity of single-walled (SWCNT) and multi- walled (MWCNT) carbon nanotubes. Based on these studies, a particular rigid MWCNT, namely Mitsui 7 (MWCNT-7), was classified as *Group 2B* (*possibly carcinogenic to humans*) [1] . Other types of CNTs (MWCNT/ SWCNT) were categorized into *Group 3*, which means they are not classifiable as to their carcinogenicity to humans. The mechanistic data regarding end-points related to lung cancer and mesothelioma, are too limited to draw conclusions [1].

Only recently, some epidemiological studies, mostly cross sectional in nature, have started providing evidence on the early biological effects of CNT in humans. A cross-sectional study by Beard et al. [2], conducted in the US in a large group of workers (*N* = 108), associated elevated blood and sputum biomarkers, like IL-18, fibrinogen, endothelin-1 and different metalloproteinases, with both exposure to CNTs and nanofibres. The conclusion was that inhalable rather than respirable CNTs were more consistently associated with biomarkers of fibrosis, inflammation, oxidative stress. Another study by Fatkhutdinova et al. [3], revealed an increase in fibrotic markers and inflammatory cytokines, in biofluids of a small group (*N* = 10) of MWCNT exposed workers compared to controls. In a subsequent study in the same population, Shvedova et al. [4] showed significant changes in expression of several key pathways, reflective of MWCNT-induced toxicity and their potential to trigger pulmonary, cardiovascular, and carcinogenic outcomes in humans. Lee et al. [5] observed and increase in oxidative stress markers in the exhaled breath condensate of exposed workers (*N* = 9). These studies support the results from *in vitro* and animal studies, stating that CNT exposure can induce oxidative stress and inflammation.

Vlaanderen et al. [6] conducted a cross-sectional study in a rather small (*N* = 22), but well characterized group of MWCNT exposed workers compared to controls (*N* = 39). They

observed increase in immune markers including basic fibroblast growth factor, and soluble IL-1 receptor II in MWCNT exposed workers. Based on the same set of workers Kuijpers et al., [7] observed an increase in a cardiovascular biomarker (endothelial damage marker intercellular adhesion molecule-1), associated with MWCNT exposure. Furthermore, Ghosh et al. [8] found differences in gene-specific DNA methylation promotor CpGs for different genes, e.g. ATM and HDAC4 in the same population.

Mitochondria, being a major source and a target of intracellular reactive oxygen species, mitochondrial DNA (mtDNA) is particularly vulnerable. We estimated mtDNA copy number by measuring the relative levels of unique mtDNA sequences of ND1 (mitochondrial encoded NADH dehydrogenase 1) gene and hmito3 (129-bp fragment) compared to nuclear human β-globin (HBG) gene. Increased mitochondrial biogenesis as an adaptive response to oxidative stress, results in an increase in mtDNA copy number (mtDNAcn; mitochondrial to nuclear genome ratio) [9]. Since ROS play a key role in the regulation of mtDNAcn [10], it serves as a potential biomarker of ROS induced mitochondrial dysfunction [9,11,12]. In addition to mtDNAcn, previous studies have also reported the influence of inflammation and oxidative stress on relative telomere length (TL) [13–16]. Telomeres consist of tandem repeats of DNA (5'-TTAGGG-3'), which play a critical role in chromosome stability and may be affected by environmental and occupational chemicals [17]. Based on previously described evidence that CNT exposure can result in elevated ROS-formation and inflammation, we hypothesize that occupational exposure to MWCNT can influence both mitochondrial function and telomeres, as reflected by the mtDNAcn and TL, respectively.

2. Study Design

The present study was designed to study the effect of MWCNT exposure at workplace on telomere length (TL) and mitochondrial copy number (mtDNAcn) in DNA isolated from peripheral whole blood. This section provides a brief overview of the study population and methods used; which has been described elaborately in the Supplementary section "Materials and Methods". The study was approved by the Commission for Medical Ethics of UZ Leuven (reference number S54607). Workers (*N*= 24) were recruited from a factory producing MWCNTs commercially and compared to 43 control subjects (no history of MWCNT exposure). Exposure assessment for MWCNT-exposed workers was performed earlier [18,19] and is described in supplementary section "M.1. Study participants and exposure assessment".

Biological sample collection was concluded in 2013, and is previously described by Vlaanderen et al. [6]. The demographic characteristics of the study population are summarized in **Table 1**. mtDNA content [11] and average relative TL [20,21] was measured using quantitative real-time polymerase chain reaction (qPCR) assay, according to methods previously published and is described in supplementary section "M.3. mtDNA copy number and TL assessment by qPCR".

<Table 1>

3. Results

3.1. Mitochondrial DNA content

In unadjusted analysis a higher mtDNAcn was observed in MWCNT exposed workers compared to controls (**Figure 1A and B**). After adjustment for age, sex, smoking behaviour, alcohol consumption and BMI, a 35.2 % (95% CI: 19.1 to 53.5 %; p<0.0001) higher mtDNAcn was observed in exposed workers, using the ND1 mitochondrial gene (Table 2). When using the mitochondrial hmito3 gene, exposed workers showed a 37.4 % (95% CI: -20.6 to 92.8%; p = 0.068) higher mtDNAc compared to non-exposed individuals.

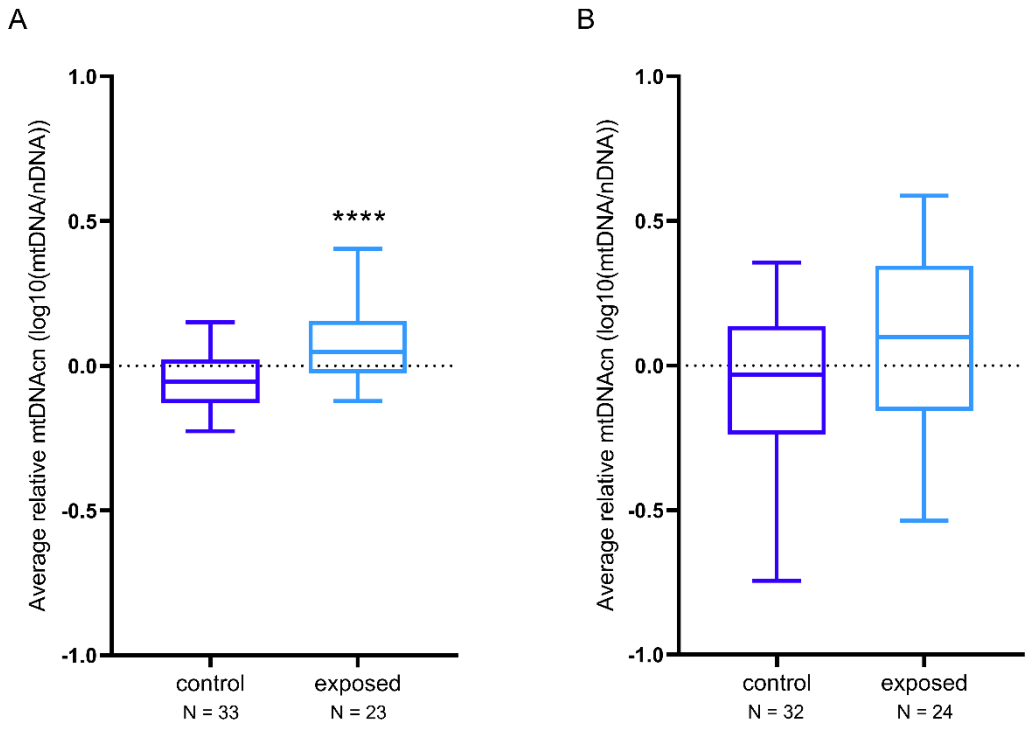

Figure 1: Box-plots showing the mtDNA content from MWCNT exposed and non-exposed controls, expressed as the log10 of (mtDNA/nDNA) for the two different mitochondrial genes, (A) ND1 and (B) hmito3; ****p < 0.0001. P-values based on unpaired t-test between exposed and control.

Besides comparing the exposed workers with the non-exposed workers, the association was examined in different groups of exposure (lab low, lab high and operator) compared with the non-exposed controls. In unadjusted (Figure 2A) and fully adjusted analysis (**Table 2**), mtDNAcn, evaluated using ND1, showed significant differences between all the groups and the non-exposed controls. A 26.5 % (95% CI: 6.4 to 50.7 %; p = 0.008) difference was found with the "lab low"-group, a 35.5 % (95% CI: 10.2 to 66.3 %; p = 0.004) difference with the "lab high"-group and a 40.6 % (95% CI: 13.8 to 73.4 %, p = 0.002) difference with the "operator"-group when compared to the non-exposed controls. For Hmito3 (**Figure 2B and Table 2**), no significant differences were found between the different groups and the non-exposed controls.

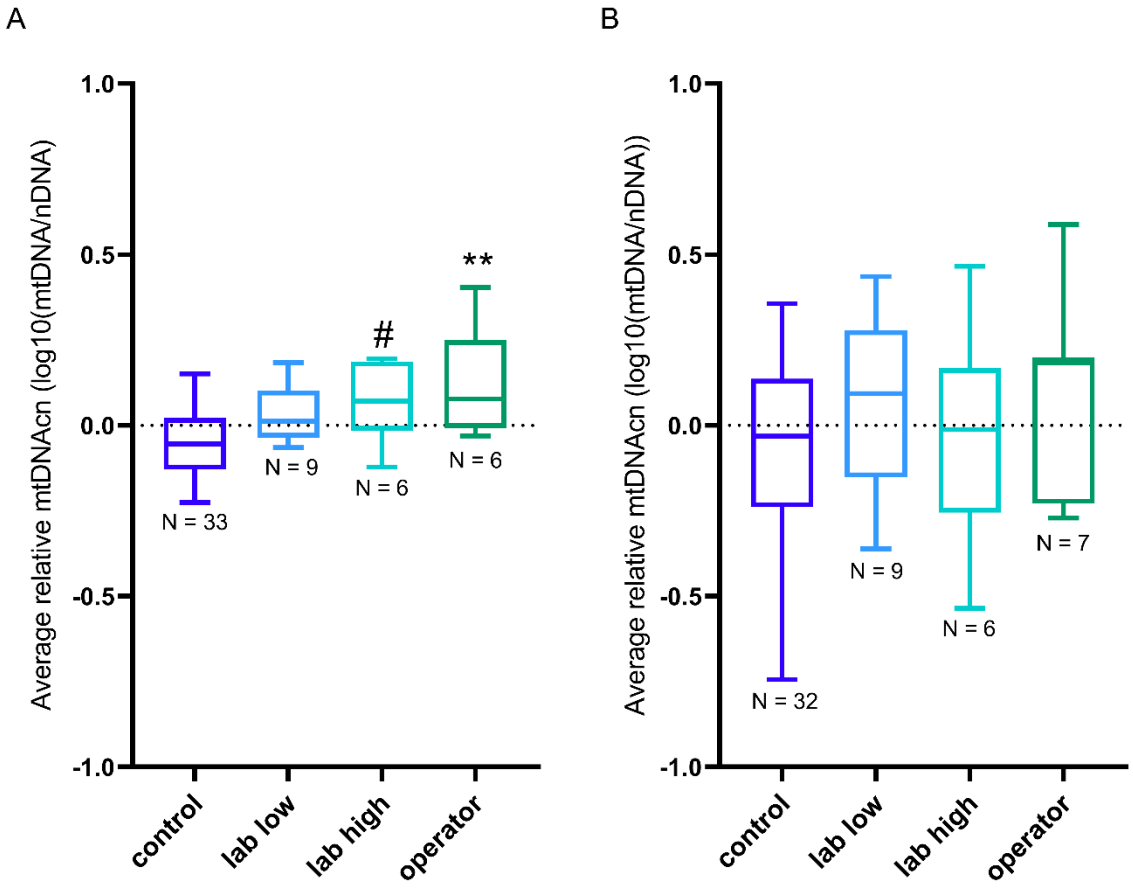

Figure 2: Box-plots showing the average relative mtDNA content (log10(mtDNA/nDNA)) from both mitochondrial genes, ND1 (A) and Hmito3 (B), for the three different MWCNT exposure groups [lab-low (1 µg/m^3 EC), lab-high (7 µg/m^3 EC), and operators (45 µg/m^3 EC)] compared with the non-exposed controls. # p < 0.10, *p < 0.05, **p < 0.01. P-values based on one-way anova between different exposure groups and the control.

3.2. Telomere length

Compared to the non-exposed controls, the MWCNT exposed workers had consistently longer telomeres (**Figure 3A and Table 2**). Workers exposed to MWCNT had 18.3 % (95% CI: 7.2 to 30.6 %; p = 0.001) longer telomeres compared to the non-exposed controls (**Table 2**). When comparing the different exposure groups (**Figure 3B and Table 2**), 27.1 % (95% CI: 10.9 to 45.5 %; p = 0.001) significantly longer telomeres were observed in the "lab low"-group, compared to the non-exposed controls. In addition, longer telomeres were also observed in the "operator"-group when compared to the non-exposed controls (18.9%; 95% CI: 1.39 to 38.99 %; p = 0.033). In the "lab high"-group no significant difference in telomere length was observed when compared to the non-exposed controls.

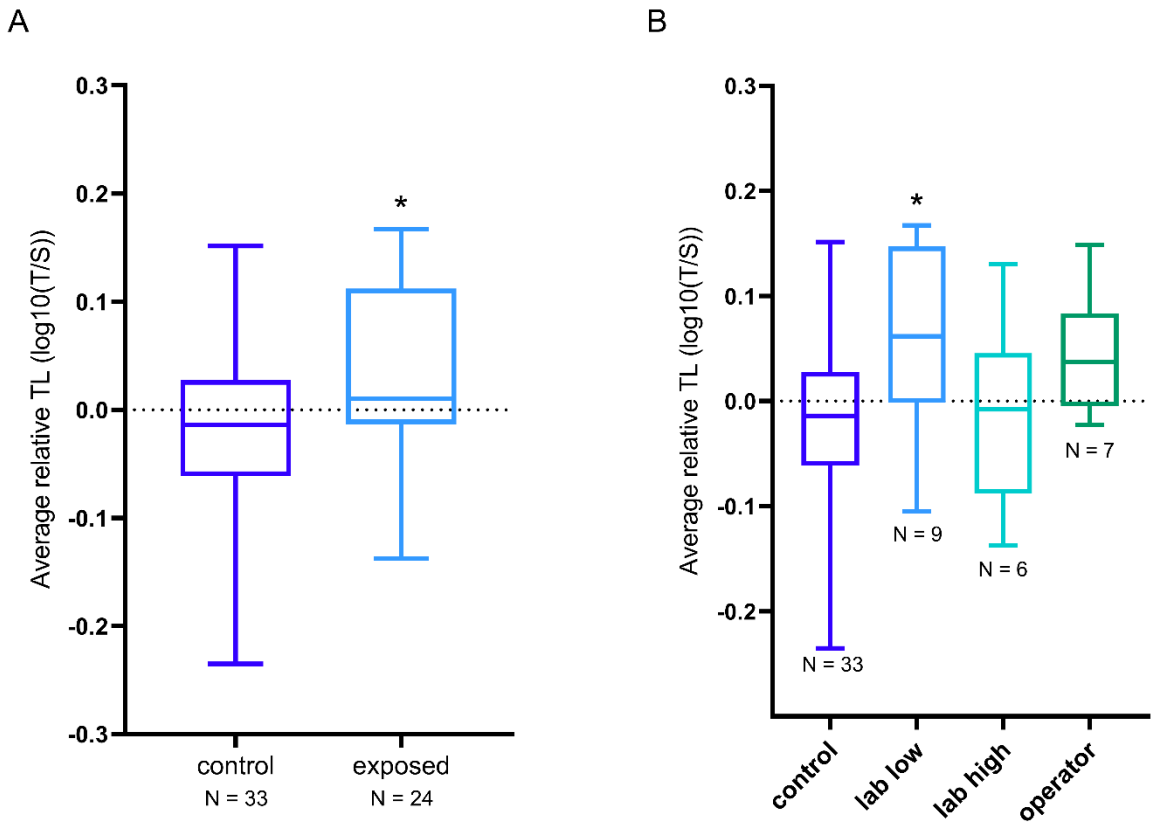

Figure 3: (A) Box-plot showing the log of the average relative TL of MWCNT exposed workers compared to non-exposed controls (B) Box-plot showing the log of the average TL for the three different groups of exposed workers [lab-low (1 µg/m^3 EC), lab-high (7 µg/m^3 EC), and operators (45 µg/m^3 EC)] compared to non-exposed controls; * p < 0.05, ** p < 0.01, *** < 0.001. P-values based on unpaired t-test (A) and one-way anova (B) between the different exposure groups and control.

4. Discussion

4.1. MWCNT exposure is associated with an increase in mtDNAcn

The first key finding of the present study is that MWCNT exposure is significantly associated with an increase in mtDNAcn when compared to non-exposed controls. This is in line with the observed increase in oxidative stress and inflammatory response in several studies [3,4], including the ones reported on the present population [7,18]. In our study, a positive association between MWCNT exposure levels and mtDNAcn was observed, when comparing three different exposure groups (lab low, lab high and operator) with the non-exposed controls. These associations were independent of the effect of age, sex, smoking behaviour, alcohol consumption and BMI.

Although the biological mechanism by which environmental exposure can induce an increase in mtDNAcn is still to be revealed, a hypothesis is proposed by Lee et al. [22]. Several studies have shown a close association between an increase in mtDNAcn and DNA damage by ROS and reduced respiratory chain function as a result of oxidative damage [23–26]. As mentioned before, the increase in mtDNAcn is thought to be a compensation for oxidative damage to mtDNA [9]. Although, whether mtDNAcn has a direct role in carcinogenesis and other pathologies/diseases is still under investigation. Concern regarding elevated mtDNAcn has been raised by several studies showing an association between an increased mtDNAcn and several cancers, e.g. head and neck cancer [27], lung cancer [28] and breast cancer [29]. Besides that, a decrease in mtDNAcn has been associated with Alzheimer's disease [30].

While there are no studies reporting mtDNAcn variation for CNT exposure, mtDNAcn variations have been observed for other exposures. A cross-sectional study by Hou et al. [12] reported higher mtDNAcn, associated with occupational exposure to PM_1, coarse particles ($PM_{2.5-10}$) and PM_{10}. Another study, conducted by Masayesva et al. [31] has shown an association between cigarette smoking and an increase in mtDNAcn in salivary cells. A study by Tan et al. [32] supports these results. Moreover, Pavanello et al. [33] reported an increase in mtDNAcn as a result of exposure to polycyclic aromatic hydrocarbons (PAHs). Lee at al. [34] has also shown this association between tobacco smoke and mtDNAcn increase in adjacent lung tissues of patients with cancer. However, several other studies reported contrary findings, e.g. a study by Janssen et al. [11] reported an inverse association between air pollution exposure and mtDNAcn in placental tissue and Pieters et al. [35] reported a decrease

in mtDNAcn associated with exposure to PAHs. Mitochondria respond dynamically to environmental insults as reflected by these studies, and depending on the exposure, exposure levels, timing, duration of exposure and study design both positive and negative associations are found. These inconsistencies potentially reflect different phases in the mitochondrial response to environmental exposures, in which both damaging and compensating mechanisms are present. Therefore, alternations in mtDNAcn, may present a biological mechanism by which CNTs, or more specific MWCNTs, affect exposed individuals.

4.2. MWCNT exposure is associated with longer telomere length

The second key finding is that humans exposed to MWCNT have significantly longer telomeres, when compared to non-exposed controls. While this is the first study on TL and CNT exposure, some studies observed longer telomeres in relation to other environmental exposures. Studies have also observed rapid increase in blood TL in response to ambient PM [36,37]. In addition, some studies report contradictory results. For example, "The Normative Aging Study" found an association between shorter telomeres in and long-term exposures to airborne particles rich in black carbon [38]. A study on the association between arsenic exposure in drinking water and TL showed longer telomeres in peripheral blood after exposure to arsenic acid [39]. They also reported a positive association between urine arsenic levels and telomerase reverse transcriptase gene (TERT) expression.

These findings suggest that carcinogenicity of arsenic among other compounds can be explained by extending the lifespan of possible malignant cells by elongation of the telomeres [39]. A similar observation was made in a Chinese female population, where an association was observed between CLPTM1L-TERT polymorphism, longer TL (measured in peripheral blood) and the risk of lung cancer [40]. A study by Jones et al. [41] also found an association between the telomerase RNA component (TERC) polymorphisms and both longer telomeres and susceptibility to colorectal cancer. This suggests that SNPs close to TERC can have functional effects on TERC expression and thus on TL. We acknowledge that only TL was evaluated but other important TL regulating factors, including telomerase activity, epigenetic factors may further explain our findings. In the context of other diseases and carcinogenesis, both positive and negative associations with TL are observed. In a study by Haycock et al. [42], associations between TL and the risk of cancer and non-neoplastic diseases were studied using a Mendelian randomization study. This study has shown that genetically increased TL is

associated with an increased risk of several cancers, e.g. melanoma, lung cancer, chronic lymphocytic leukaemia, which has been confirmed by several other prospective observational or Mendelian randomization studies [43,44]. It is important to know that these results should be interpreted as a reflection of the average association at the population level. However, these findings are contradictory to those based on retrospective studies, tending to report an association between shorter telomeres and an increased risk for cancer [45,46]. A plausible explanation, proposed by Aviv et al., [47], for the relation between increased TL and cancer, is the potential accumulation of mutation, due to telomere lengthening associated stem-like properties, similar to the findings of Haycock et al. [42]. Although it is not possible to connect our findings with an increased risk in cancer, it must be said that some studies show an association between long somatic telomeres and some forms of cancer.

We also would like to recognize some strengths and limitations of our study. First, we have a small-sized study and our results need to be confirmed in a larger independent investigation. Nevertheless, we observed robust associations independent of the effect of age, sex, smoking behaviour, alcohol consumption, BMI. We have a well characterized exposure group. Finally, the measurements are conducted only at one time point and therefore a follow-up of the same subject would provide a better interpretation of the present findings.

5. Conclusion

Overall, a higher mtDNAcn and longer telomeres were observed in MWCNT exposed workers when compared to non-exposed controls, independent of age, sex, smoking behavior, alcohol consumption and BMI. When comparing the three groups of exposure (lab low, lab high and operator), significant exposure-associated differences were observed between the groups. While we observe significant change in mtDNAcn and TL in the MWCNT exposed workers, no association can be made regarding possible disease outcome at the moment.

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Table 1: Demographic characteristics of the study population reported as N, (%) and mean ± SD.

Variables		**Control (*N* = 43)**	**Exposed (*N* = 24)**	**P-value**
Sex	Male	32 (74.4 %)	20 (83.3 %)	0.409
	Female	11 (25.6 %)	4 (16.7 %)	
Age (years)		34.6 ± 8.57	35.9 ± 6.90	0.729
BMI (kg/m^2)		24.88 ± 4.71	27.19 ± 4.99	0.064
Smoking	Never smoker	24 (55.8 %)	13 (54.2 %)	0.805
	Former smoker	7 (16.3 %)	6 (25.0 %)	
	Current smoker	12 (27.9 %)	5 (20.8 %)	
Alcohol consumption	Yes/No	35/8	15/9	0.091
	Glasses/day [b]	1.1 ± 0.91	0.9 ± 0.77	
Previous history of exposure to chemicals [a]		9 (20.9 %)	8 (33.3 %)	
Duration of exposure to nanoparticle at current job (years)		/	4.25 ± 2.40	

[a] as reported by the study subjects.

[b] Alcoholic drinks consumed on average per day over the past 4 weeks.

Table 2: Association of mtDNA and TL with CNT exposure.

	mtDNA (ND1)			mtDNA (hmito3)			TL		
	n	**% difference (95%CI)**	**P-value**	**n**	**% difference (95%CI)**	**P-value**	**n**	**% difference (95%CI)**	**P-value**
MWCNT exposure									
Non-exposed controls	33	Ref		32	Ref		33	Ref	
MWCNT exposed	23	35.2 (19.1, 53.5)	<0.0001	24	37.4 (-20.6, 92.8)	0.068	24	18.3 (7.2, 30.6)	0.001
Detailed exposure groups									
Non-exposed controls	33	Ref		32	Ref		33	Ref	
Lab low (1 μg/m^3 EC)	9	26.5 (6.4, 50.7)	0.008	9	23.0 (-22.6, 95.9)	0.38	9	27.1 (10.9, 45.6)	0.001
Lab high (7 μg/m^3 EC)	6	35.5 (10.2, 66.3)	0.004	6	8.9 (-37.2, 88.8)	0.76	6	8.4 (-7.7, 27.4)	0.33
Operator (45 μg/m^3 EC)	6	40.6 (13.8, 73.4)	0.002	7	61.4 (-5.6, 176.7)	0.081	7	18.9 (1.4, 39.0)	0.033

[a]Estimates from the linear regression models provided as a % difference (95%CI) in outcome compared with the non-exposed group (Ref); MWCNT- Multi-walled carbon nanotube; relative telomere length (TL), mitochondrial copy number (mtDNAcn); ND1 (mitochondrial encoded NADH dehydrogenase 1); Hmito3 (Human mitochondrial genome); Models adjusted for age, sex, smoking behaviour, alcohol consumption and BMI