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Ahmed Karoui, Clément Crochemore, Paul Mulder, David Preterre, Fabrice Cazier, et al.. An integrated functional and transcriptomic analysis reveals that repeated exposure to diesel exhaust induces sustained mitochondrial and cardiac dysfunctions. *Environmental Pollution*, 2019, 246, pp.518-526. 10.1016/j.envpol.2018.12.049 . hal-01978723

HAL Id: hal-01978723

<https://univ-rennes.hal.science/hal-01978723>

Submitted on 14 Jan 2019

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An integrated functional and transcriptomic analysis reveals that repeated exposure to diesel exhaust induces sustained mitochondrial and cardiac dysfunctions

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1 Abstract

2 Diesel exhaust (DE) contributes to air pollution, an important risk factor for cardiovascular
3 diseases. However, the mechanisms by which DE exposure induces cardiovascular
4 dysfunction remain unknown and there is still debate on the contribution of the primary
5 particulate matter (PM) fraction compared to the gaseous phase. Although the mitochondria
6 play a key role in the events leading to cardiovascular diseases, their role in DE-induced
7 cardiovascular effects has not been investigated. The aim of this study was to highlight
8 cardiac and mitochondrial events that could be disrupted following acute and/or repeated DE
9 exposures and the contribution of gaseous pollutants to these effects. To address this question,
10 Wistar rats were exposed to DE generated under strictly controlled and characterized
11 conditions and extracted upstream or downstream of the diesel particulate filter (DPF).
12 Evaluation of the cardiac function after acute DE exposure showed a disturbance in
13 echocardiographic parameters, which persisted and worsened after repeated exposures. The
14 presence of the DPF did not modify the cardiovascular dysfunction revealing an important
15 implication of the gas phase in this response. Surprisingly, redox parameters were not altered
16 by DE exposures while an alteration in mitochondrial oxidative capacity was observed.
17 Exploration of the mitochondrial function demonstrated a more specific alteration in complex
18 I of the respiratory chain after repeated exposures, which was further confirmed by
19 transcriptional analysis of left ventricular (LV) tissue. In conclusion, this work provides new
20 insights into cardiovascular effects induced by DE, demonstrating a cardiac mitochondrial
21 impairment associated with the gaseous phase. These effects suggest deleterious
22 consequences in terms of cardiac function for vulnerable populations with underlying energy
23 deficit such as patients with heart failure or the elderly.

24 **KEYWORDS:** diesel exhaust, particles, cardiovascular, mitochondria, gene expression

25

26 GRAPHICAL ABSTRACT (adapted from (Douki et al., 2018)

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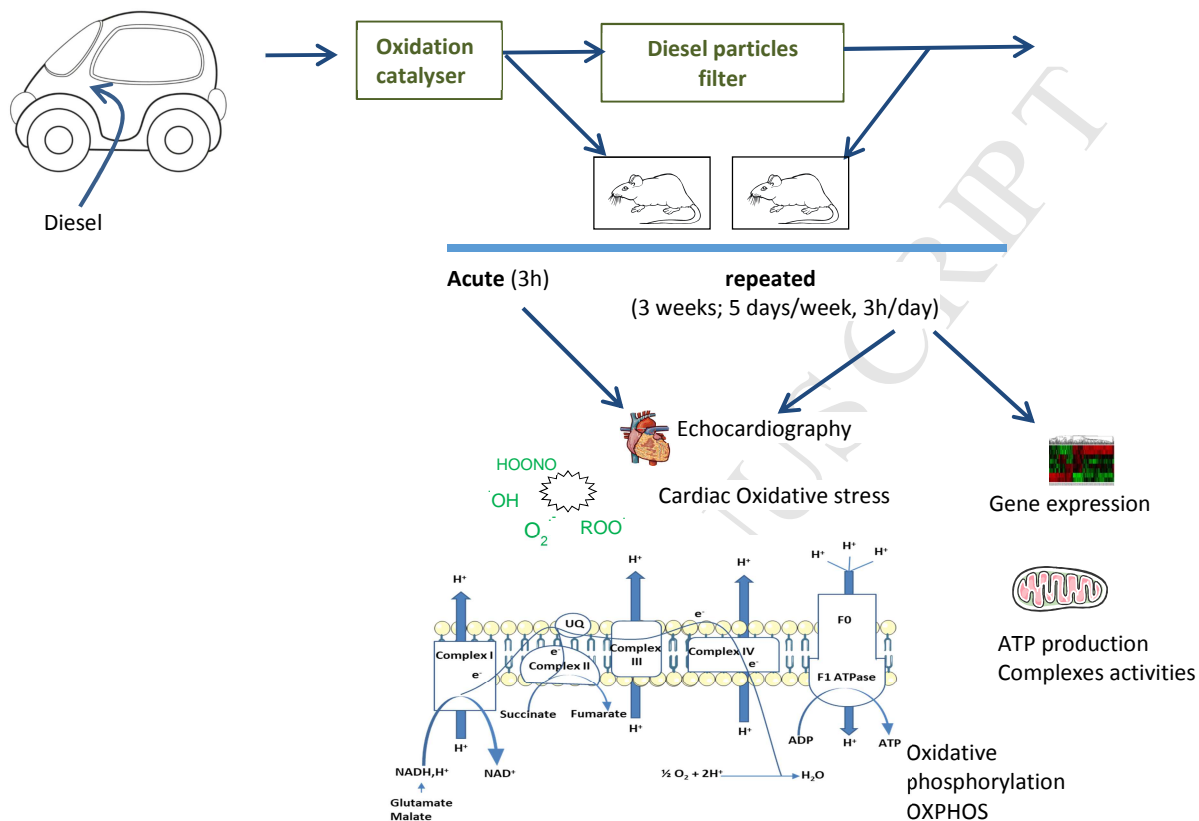
35

36 CAPSULE

37 Diesel exhaust induces an acute cardiovascular response, which leads to a sustained cardiac
 38 mitochondrial defect and cardiac dysfunction after repeated exposures.

39 HIGHLIGHTS

- 40 - Acute and repeated diesel exhaust (DE) exposures induced a cardiac dysfunction in
 41 rats
- 42 - Repeated DE exposures induced a decrease in OXPHOS capacity
- 43 - OXPHOS defect is associated with a decrease in complex I activity



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- 42 - Repeated DE exposures induced a decrease in OXPHOS capacity
- 43 - OXPHOS defect is associated with a decrease in complex I activity

- 44 - Both whole DE and gaseous phase contribute to these cardiac and mitochondrial
45 defects
- 46 - Parameters related to cardiac oxidative stress are not affected in these conditions.

ACCEPTED MANUSCRIPT

47 1. INTRODUCTION

48 Air pollution is a major environmental risk to health and resulted in almost 9 million
49 premature deaths in 2015 (Burnett et al., 2018). Fuel combustion contributes 85% of the
50 particulate pollution and almost all the nitrogen oxide and sulphur (Landrigan et al., 2018).
51 Vehicular exhaust, including diesel exhaust (DE), are responsible for a large part of the air
52 pollution, and epidemiologic data link particulate matter (PM) levels to adverse
53 cardiovascular effects, for both acute events (Link et al., 2013; Ye et al., 2016) and chronic
54 exposures (Mordukhovich et al., 2015; Puett et al., 2009). It is well-documented that PM air
55 pollution is associated with a large number of cardiovascular effects such as endothelial
56 dysfunction (Krishnan et al., 2012; Pope et al., 2016), increased blood pressure (Honda et al.,
57 2018; Ibaldo-Mulli et al., 2001), accelerated arterial thrombosis (Nemmar et al., 2003),
58 autonomic imbalance and arrhythmias (Carll et al., 2012; Folino et al., 2017).

59 Given the growing concern about the health effects of particulate emissions from DE, diesel
60 engine and fuel technology advances have been made to respond to the evolution of emission
61 regulations. These advances resulted in changes to the composition of DE with, for example,
62 lower particulate mass (less than 1%) with the use of diesel particulate filters (DPF)
63 (McClellan et al., 2012). These changes in DE compositions are likely to have an impact on
64 the cardiovascular effects of diesel engine exhaust inhalation. However, a limited number of
65 studies have investigated the impact of these new emissions on the cardiovascular responses.
66 The Advanced Collaborative Emissions Study (ACES) analysed new DE technology in
67 healthy rats exposed for 24 months in order to assess plasma markers of vascular
68 inflammation, thrombosis, cardiac fibrosis and aorta morphometry and the results showed
69 limited effects (Conklin et al., 2015). In human, compared to untreated DE exposure, DE
70 exposure in the presence of a DPF has been associated with a lack of acute cardiovascular
71 effects (impaired vasodilation and ex-vivo thrombus formation) (Lucking et al., 2011). These

72 results agree with the acute beneficial effects of reducing particle emissions from diesel
73 engines. However, in another study, Karthikeyan et al. (Karthikeyan et al., 2013)
74 demonstrated that catalyzed DPF exhaust resulted in heightened injury and inflammation due
75 to an increase in nitrogen dioxide emissions and the release of ultrafine particles. These
76 results outline the need for further toxicological assessments in order to understand the
77 underlying mechanisms and identify the specific components that induce health effects.

78 Several biological mechanisms behind the DE-induced cardiovascular effects have previously
79 been suggested and are generally assumed to play a key role, such as a disturbance of the
80 systemic autonomic nervous system, translocation of PM or particles compounds into the
81 systemic circulation and pulmonary oxidative stress (Brook et al., 2010). At the cellular level,
82 the well-described induction of a pulmonary oxidative stress and inflammation observed after
83 DE, could induce the release of pro-inflammatory mediators that may alter the vascular
84 function (Channell et al., 2012). Several studies have also pointed out the existence of
85 markers of myocardial oxidative stress after DE particulate exposures (Robertson et al., 2014;
86 Yokota et al., 2008). These markers often reflect an early induction of genes involved in the
87 antioxidant response, suggesting rapid adaptive responses (Gurgueira et al., 2002).

88 More recently, mitochondria have emerged as a target of air pollutants, for example after
89 sulphur dioxide (Qin et al., 2016), carbon monoxide (Reboul et al., 2017) or mining PM
90 (Nichols et al., 2015) exposures. In addition, mitochondrial dysfunction is associated with
91 many cardiovascular diseases, given their central role in a large number of cellular functions
92 such as reactive oxygen species generation and ATP production, as well as apoptosis.
93 Consequently, mitochondria could play a key role in the events leading to cardiovascular
94 diseases due to air pollutants. However, whether diesel exhaust is associated with cardiac
95 mitochondrial dysfunction and/or an oxidative stress and contributes to cardiac dysfunction
96 has not yet been investigated.

97 Therefore, the aims of the present study were to test whether a cardiac oxidative stress and/or
98 a mitochondrial defect precede cardiovascular dysfunction after DE exposure and whether the
99 gaseous pollutants contribute to these effects. To achieve these objectives, we exposed
100 animals to dilute exhaust emitted from a diesel motor that was operated under driving cycles,
101 upstream or downstream of a DPF, and after acute or repeated exposures to evaluate
102 immediate and sustained effects.

103 **2. EXPERIMENTAL PART**

104 *2.1. Diesel exhaust (DE) generation and emission characterization*

105 DE were derived from a supercharged common rail direct injection diesel engine equipped
106 with a diesel oxidation catalyst, and located upstream (P1) or downstream (P2) of a DPF, as
107 previously described (Douki et al., 2018). The diesel engine was placed in a test bench cell
108 equipped with a dynamic asynchronous chassis dyno, which allows continuous control of
109 both engine speed and load, as well as the recording of technical parameters. The engine was
110 operated on commercial low sulphur diesel (less than 3 ppm of sulphur), and used under
111 dynamic conditions according to the “New European Driving Cycle” (NEDC). The
112 concentrations of pollutants were monitored as described in the supplementary experimental
113 part and according to previous published methods (Caplain et al., 2006)(Cazier et al., 2016).

114 *2.2. Exposure study design*

115 This project was reviewed and approved by a certified committee according to European
116 legislation (authorization number 00291.01). The experiments were performed on adult male
117 Wistar rats (275-300 g, Janvier Inc., Le Genest Saint Isle, France). For inhalation exposures,
118 DE were directly drawn from the exhaust line, directed by a Dekati Fine Particle sampler
119 (FPS) (Dekati Finland) and diluted by a factor 10 as previously described (Douki et al., 2018).
120 Rats were placed in whole body inhalation chambers as previously described (Anselme et al.
121 2007). All DE exposures were conducted from the exhaust after dilution as described above,

122 upstream (P1) or downstream (P2) of the DPF. For repeated exposures, the animals were
123 exposed for 3 h/day, 5 days/week during 3 weeks and the biological evaluations were
124 performed after a 16h recovery period in clean air. For acute evaluations, rats were exposed
125 for a single 3 h period and the evaluations were performed after a recovery period of 1h. This
126 study design is summarized in a scheme presented in the supplementary material and method
127 section.

128 *2.3. Echocardiographic assessments*

129 Echocardiographic assessments were performed in sedated rats (100 mg/kg ketamine; 3
130 mg/kg xylazine; Easote AU5 Advanced Ultrasonography) after the recovery periods. Cardiac
131 ventricular dimensions were measured using M-mode tracings recorded from a 2-dimensional
132 short-axis view at the level of the papillary muscles. Echocardiography provided
133 measurements of LV end-diastolic (LV_{edd}) and end-systolic (LV_{esd}) diameters and posterior
134 wall thickness at diastole (PW_{EDT}) and at systole (PW_{EST}). Relative wall thickness (RWT)
135 was calculated as $2 \times PW_{EDT} / LV_{edd}$. LV systolic function was assessed by the fractional
136 shortening $[(LV_{edd} - LV_{esd}) / LV_{edd}] \times 100$. In addition, velocity-time integral was measured
137 by pulsed-wave Doppler, and cardiac output (CO) was calculated as $CO = \text{aortic velocity-time}$
138 $\text{integral} \times [(\pi \times \text{LV outflow diameter})^2 / 4] / 100 \times \text{heart rate}$ (Moritz et al., 2003).

139 *2.4. Cardiac mitochondrial assessments*

140 After echocardiographic assessments, rats were euthanized (0.2 g/kg sodium thiopental), the
141 heart was removed from the chest and the left ventricle (LV) was dissected on ice and
142 weighted. A part of the LV was freshly used for the measurement of mitochondrial oxidative
143 phosphorylation capacity (OXPHOS) and ATP production and the rest was frozen into liquid
144 nitrogen for the mitochondrial enzymatic assays.

145 *Oxygen consumption*

146 We assessed OXPHOS in cardiac permeabilized fibers prepared as described previously
147 (Veksler et al., 1987, Vergeade et al., 2010). Oxygen consumption was measured at 22°C,
148 using a Clark electrode (Strathkelvin Instruments, Scotland, UK), in a respiration buffer
149 consisting of 2.77 mM CaK₂ EGTA (2.77 mM EGTA, 2.77 mM CaCO₃ and 5.54 mM KOH),
150 7.23 mM K₂EGTA (100 nM free Ca²⁺), 1.38 mM MgCl₂ (1 mM free Mg²⁺), 20 mM taurine,
151 90 mM potassium methanesulfonate, 20 mM imidazole, 10 mM sodium methane sulfonate,
152 and 2 mg/ml BSA, pH 7.1. After a stabilization period, O₂ consumption rates were recorded
153 with 2 mM ADP, 10 mM glutamate and 4 mM malate as mitochondrial substrate (VGM). To
154 evaluate oxygen consumption from complex II, complex I was blocked with 2 mM amytal
155 and 10 mM succinate were added (VS). Respiration rates are expressed per mg of proteins of
156 cardiac fibers.

157 ATP production

158 The ATP production was evaluated from isolated myocardial subsarcolemmal (SSM) and
159 interfibrillar mitochondria (IFM), according to previously described protocols (Palmer et al.,
160 1977; Vergeade et al., 2010) as detailed in the supplementary material and methods.

161 Mitochondrial enzymatic activities

162 The activities of NADH-ubiquinone oxidoreductase (complex I), ubiquinol cytochrome c
163 reductase (complex III), cytochrome c oxidase (complex IV) and citrate synthase activities
164 were assayed in LV homogenates using established methods (Spinazzi et al., 2012) and
165 described in the supplementary material and methods.

166 *2.5. Parameters of oxidative stress*

167 Cardiac oxidative stress was evaluated from LV homogenates by the measurements of
168 antioxidant enzymes activities and glutathione redox state, as previously described (Moritz et
169 al., 2003).

170 *2.6. Transcriptomic analysis*

171 RNA was isolated from frozen left ventricle (LV) using RNeasy Plus mini kit (Qiagen,
172 France) following manufacture's protocol. Quantity and quality of the RNA were measured
173 using a Nanodrop spectrophotometer and the Agilent 2100 Bioanalyser (Agilent technologies,
174 Santa Clara, CA, USA). RNA with a RNA Integrity Numbers (RIN) higher than 7.8 were
175 used for reverse transcriptase. For hybridization, gene expression was assessed using one chip
176 per LV; 6 replicates from each of the 3 groups exposed to filtered air, repeated exposures of
177 DE upstream (P1) and downstream (P2) DPF, were further processed as follows for
178 GeneChips analysis. The gene expression profiles were determined using GeneChip®
179 RAGENE 2.0 ST Arrays (> 24 000 genes, Affymetrix) through the genomic platform of
180 Hospital Cochin (University Paris Descartes). Samples were hybridized onto array chips,
181 stained, washed, and scanned according to Affymetrix protocol. The array image and cell
182 intensity files (.CEL files) were generated by Affymetrix GeneChip Command Console. After
183 the normalization of the data using global scale normalization, 2 chips were eliminated and
184 further analysis was realized on 28 chips. Resulting signal intensities were on log₂-scale.

185 *2.7. Quantitative real-time PCR*

186 Total RNA were subjected to reverse transcription using RT Applied Biosystem kit
187 (Courtaboeuf, France). qPCR assays were next performed using Power SYBR Green PCR
188 master kit according to the manufacturer's instructions (Life Technologies) and an ABI 7900
189 detector (Applied Biosystem). Kicqstart gene-specific primers were purchased from Sigma-
190 Aldrich (St Quentin Fallavier, France). Amplification curves of the PCR products were
191 analyzed with the ABI Prism SDS software using the comparative cycle threshold method.
192 The relative gene expression was calculated by using the $\Delta\Delta C_T$ analysis for each sample after
193 normalization against β -actin gene expression. The control air-exposed rats served as a
194 reference and their mRNA expression was arbitrarily considered as 1 unit for each analyzed
195 gene.

196 *2.8. Statistical analysis*

197 For biological parameters, results are expressed as mean \pm sem. One-way ANOVA was used
198 to compare the effects of DE exposures, followed by the post-hoc Tukey test where
199 appropriate (normal distribution verified by the Shapiro-Wilk test) or by Kruskal-Wallis test,
200 followed by Dunn's multiple comparison post-test. Differences were considered statistically
201 significant when $p < 0.05$.

202 3. RESULTS

203 3.1. Diesel exhaust characterization

204 The exhaust characterization was performed from raw exhaust in both upstream (P1) and
205 downstream (P2) of the DPF, during the NEDC cycles and the results for regulated pollutants
206 were presented in the Table S1. The DPF reduced the total PM concentrations (P2). Cold start
207 induced high emissions of CO and total gaseous hydrocarbons whereas NO₂ emissions were
208 increased during driving conditions. Based on these measurements, we consider that after
209 dilution, the concentrations of pollutants to which rats were exposed in P1 were 2.5 mg
210 PM/m³ for the mean with a median at 1.5 mg/m³. For NO₂, the rats were exposed to an
211 average level of 3 ppm after the first NEDC cycle.

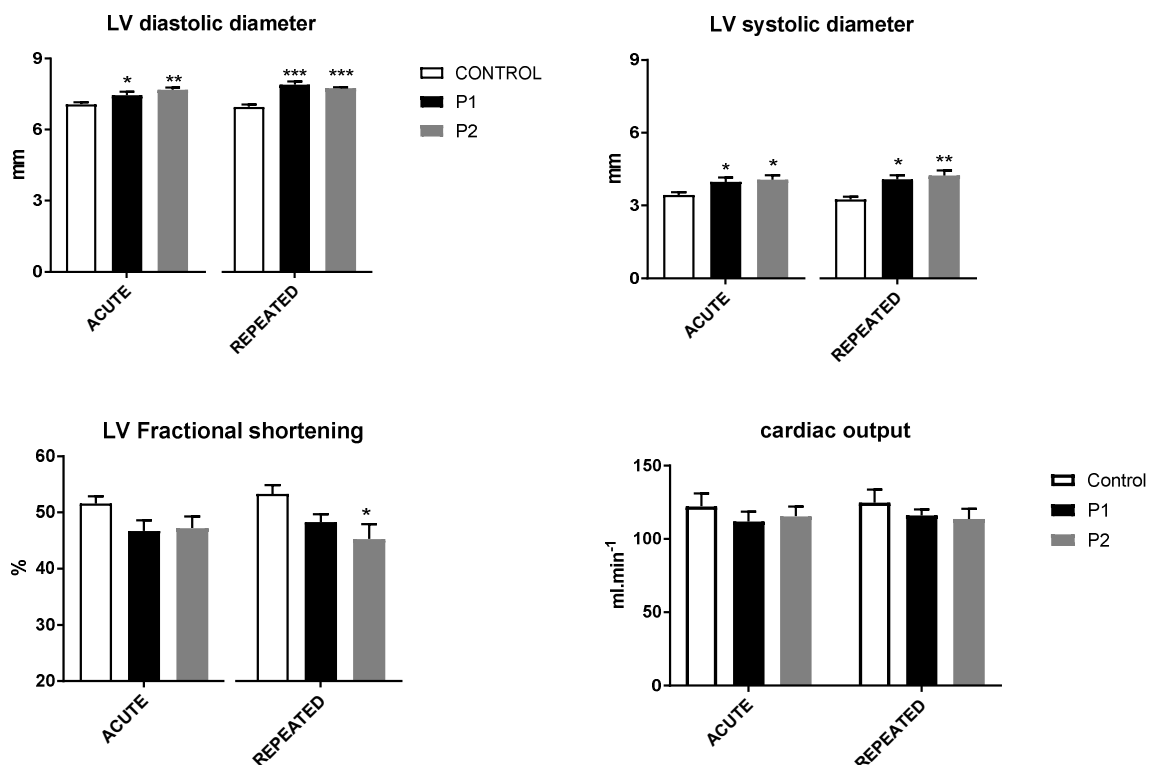
212 Concentrations of aldehydes and mono-aromatic volatile organic compounds (VOCs) were
213 measured two times, on two different days randomly selected during the study (Table S2).
214 The total aldehyde concentrations measured upstream and downstream of the DPF are quite
215 homogeneous during the two days. Formaldehyde and acetaldehyde are the main carbonyl
216 compounds detected. An increase by a 4-fold factor of the total carbonyl compound
217 concentration is noted downstream of the DPF (P2). The total mono-aromatic VOC
218 concentration decreases by a ten-fold downstream of the DPF (P2) (Table S2).

219 For alkanes (Fig. S1) and PAH (Fig. S2), a decrease of the total concentrations is observed in
220 P2. The main alkanes are between C15 and C30 in P1, whereas in P2 the concentrations of
221 alkanes heavier than C25 strongly decrease (Fig. S1). Naphtalene represents more than 80%
222 of the detected PAH and shows a strong decrease (nearly 70%) after the DPF. The other PAH
223 detected are phenanthrene, fluoranthene and pyrene. No heavier PAH is detected due to the
224 new engine generation with high pressure injection, which optimizes the fuel combustion and
225 thus decreases the heavy PAH generation (Fig S2).

226

227 3.2. Cardiovascular function following diesel exposure.

228 After acute DE exposure, a slight but significant increase in both LV end-diastolic (+5,
 229 $p < 0.05$ and +8%, $p < 0.01$, P1 and P2 respectively, vs. control) and end-systolic diameters (+16
 230 and +18%, $p < 0.05$ P1 and P2 respectively, vs. control) was observed (Fig 1). This increase
 231 translates a limited dilation of ventricular chambers. This impairment persisted and worsened
 232 after repeated exposures, with an increase of about 30% in both LV end-diastolic ($p < 0.001$)
 233 and end-systolic ($p < 0.01$) diameters vs. control and a decrease in fractional shortening by
 234 15% downstream of the DPF, whereas cardiac output was not modified (Fig. 1). Rats exposed
 235 to DE also exhibited a decrease in posterior wall thickness at systole and diastole after 3
 236 weeks of exposure (Table S3).



237

238 **Figure 1:** Effect of DE on echocardiographic parameters after acute or repeated exposures.

239 Rats were exposed to DE derived upstream (P1) or downstream (P2) of the DPF. * $p < 0.05$,

240 ** $p < 0.01$, *** $p < 0.001$ vs Control (n = 6-10 rats)

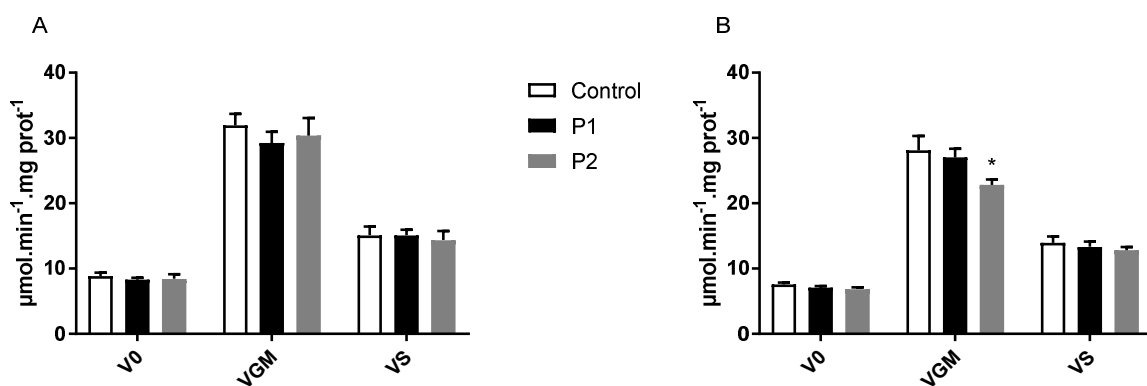
241 3.3. Markers of Cardiac oxidative stress

242 Acute and repeated exposures to DE did not cause any statistically significant changes
 243 in myocardial antioxidant enzymatic activities and GSH redox ratio. Aconitase activity, a
 244 highly vulnerable enzyme to oxidative stress, remained unchanged, whatever the experimental
 245 conditions (Table S4).

246

247 3.4. Mitochondrial function following diesel exposure

248 To investigate mitochondrial function after DE exposure, the oxygen consumption of
 249 permeabilized cardiac fibers was measured (Fig.2). Although there was no difference after
 250 acute exposure (Fig. 2A), we found that repeated exposure significantly decreased oxygen
 251 consumption specifically in P2, with glutamate and malate (VGM) as substrates (Fig. 2B).

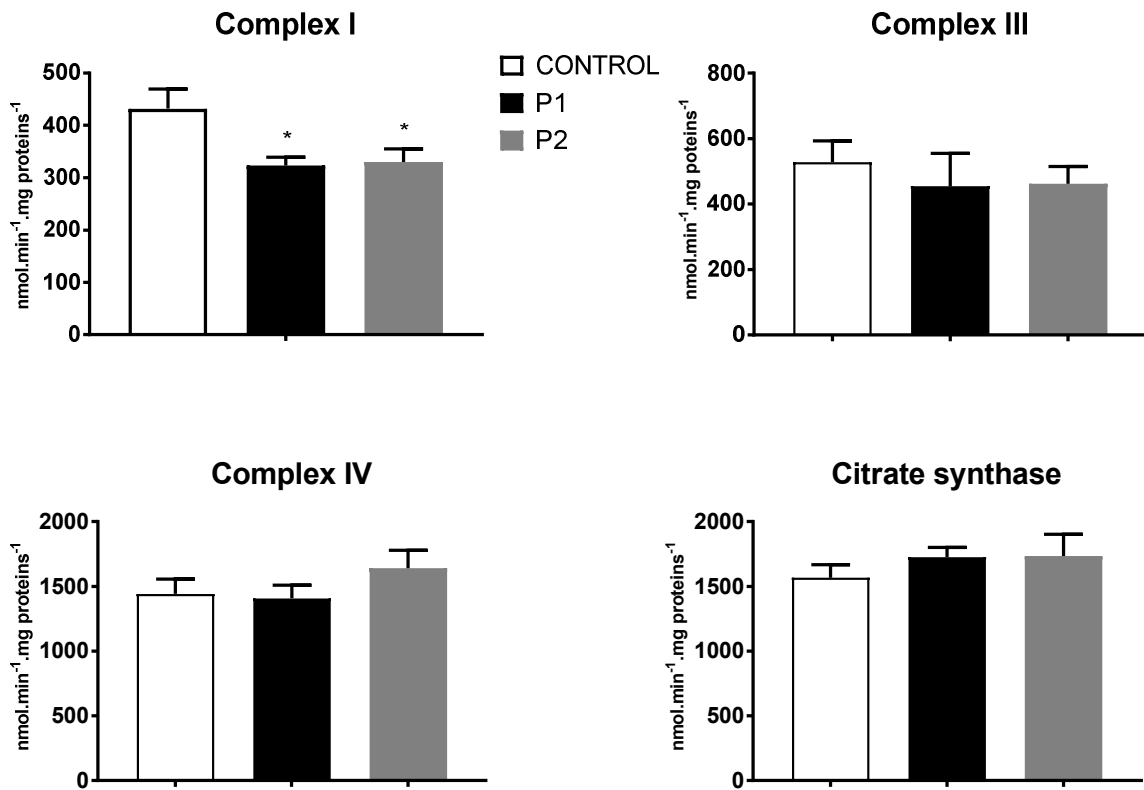


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253 **Figure 2:** Respiration in LV skinned cardiac fibers after acute or repeated exposures. Basal
 254 respiration rate (V0), complex I respiration with glutamate and malate (VGM), complex II
 255 respiration with succinate (VS) were evaluated in Control, P1 and P2 groups, after acute (A)
 256 or repeated exposures (B). * $p < 0.05$ vs Control (n=6-10 rats in duplicate).

257 To further investigate the effects of repeated DE exposure on mitochondrial OXPHOS, the
 258 functional activities of mitochondrial respiratory chain complexes I, III and IV were analyzed
 259 (Fig. 3). Complex I activity decreased by about 25% in both upstream and downstream of the

260 DPF, compared with the control group, whereas no significant changes were found for
 261 complex III or complex IV activities. Activity of citrate synthase, a mitochondrial-specific
 262 matrix protein, was not significantly different between the groups.



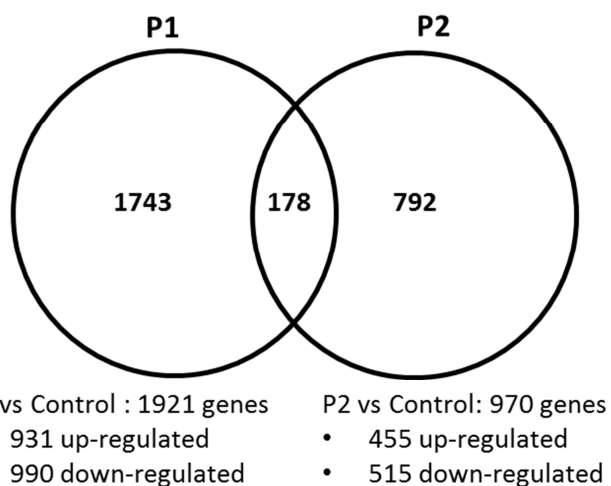
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264 **Figure 3:** Mitochondrial enzymatic activities in LV after repeated exposures. Enzymatic
 265 activities were evaluated in LV homogenates from Control, P1 and P2 groups. *p<0.05 vs.
 266 Control (n=8 rats)

267 To complete the assessment of mitochondrial function, we next measured ATP production
 268 rates in freshly isolated mitochondrial fractions, of exposed rats during 3 weeks. For this
 269 evaluation, we performed the isolation of SSM and IFM after proteinase treatment. We found
 270 that ATP production was reduced specifically in the IFM fractions after repeated DE exposure
 271 compared with the control group, whereas there was no change in ATP production in the SSM
 272 fractions (Fig. S3).

273 3.5. Transcriptomic alterations

274 A wide genomic analysis of DE effects on LV was performed considering a p-value Log-
 275 Ratio < 0.05. Figure 4 represents the number of genes regulated after repeated exposure of
 276 DE. It reveals that 1921 and 970 genes were identified as regulated in P1 vs Control and P2 vs
 277 Control, respectively, thus showing that the presence of DPF reduces by half the number of
 278 genes regulated by DE. Among the DE-regulated genes, we found that about 50% of genes
 279 are up- or down-regulated both for P1 vs. Control and P2 vs. Control (Fig.4). Venn diagram
 280 also reveals that only 178 responsive genes are common to P1 and P2, when compared to
 281 control, corresponding to 9.2 % and 18.3% of genes from P1 vs. Control and P2 vs. Control,
 282 respectively.



284 **Figure 4:** Number of DE-regulated genes detected by microarray in LV after repeated
 285 exposure. Venn diagrams illustrate the number of genes regulated in P1 and P2 when
 286 compared to control. Genes were selected based on a pValue LogRatio < 0.05 (n=6 rats)

287
 288 The lists of genes corresponding to P1 vs Control (1921 genes), to P2 vs Control (970 genes)
 289 and to genes in common in both groups (178 genes) can be found as supplementary tables
 290 (Tables excel S5, S6 and S7). As previously described by our laboratory on lung tissue after
 291 repeated exposure to DE (Douki et al, 2018), a high proportion of genes identified as DE-

292 responsive corresponds in fact to slightly regulated genes. Thus, after applying a cut-off at 1.3
293 fold change factor to avoid the impact of differentially expressed genes with very small
294 change (less than 1.3-fold), we found that the numbers of genes regulated by DE vs control
295 were 234 (representing 12.1% of 1921 genes) and 46 (representing 4.7% of 970 genes) for
296 exposure conditions upstream (P1) and downstream (P2) of DPF, respectively (Tables S5 and
297 S6).

298 Concerning the common genes, we identified that 21 genes were regulated by a factor ≥ 1.3
299 fold and, among them, 10 genes were up-regulated and 11 genes were down-regulated by DE
300 exposure (Table S7).

301 Next, we have submitted to the Ingenuity Pathway Analysis (IPA) software (Ingenuity
302 Systems, Mountain View, CA) the P1 (1921 genes) and the P2 (970 genes) gene lists. The 5
303 top canonical pathways and the 5 top Tox lists for P1 vs Control and P2 vs Control are
304 represented in the Table S8. IPA analysis reveals that pathways related to “mitochondria”
305 appear several times in the P1 group vs. control (*mitochondrial dysfunction*, *Increases*
306 *permeability transition of mitochondria and mitochondrial membrane* and *Oxidative*
307 *phosphorylation*) and one time in the P2 group vs. Control (*Swelling of mitochondria*),
308 demonstrating the importance of DE effects on mitochondria in LV tissue. Among the genes
309 from the *mitochondrial dysfunction* and *Oxidative phosphorylation* pathway, several
310 corresponded to genes coding for mitochondrial complex I subunits (.i.e. NDUFA7,
311 NDUFB6, NDUFC1...); therefore, we analyzed the level of expression of some of these
312 genes by qPCR. Our results showed that NDUFA7 mRNA expression was significantly up-
313 regulated in LV from rats exposed to DE in P1 when compared to control rats and to rats
314 exposed to DE in P2 (Fig.5). By contrast, the levels of mRNA expression of genes coding for
315 other subunits of mitochondrial complex I were not significantly modified by repeated DE
316 exposure (Fig.5). Such an absence of significance may be related to the dispersion of mRNA

317 expression of these genes observed in the group of rats exposed to DE in P1 and also, to the
 318 weak modulation factors found in microarrays, ranging between -2.1 to 1.9 fold, as previously
 319 described in a similar study realized on lung tissue after DE repeated exposures (Douki et al.,
 320 2018).

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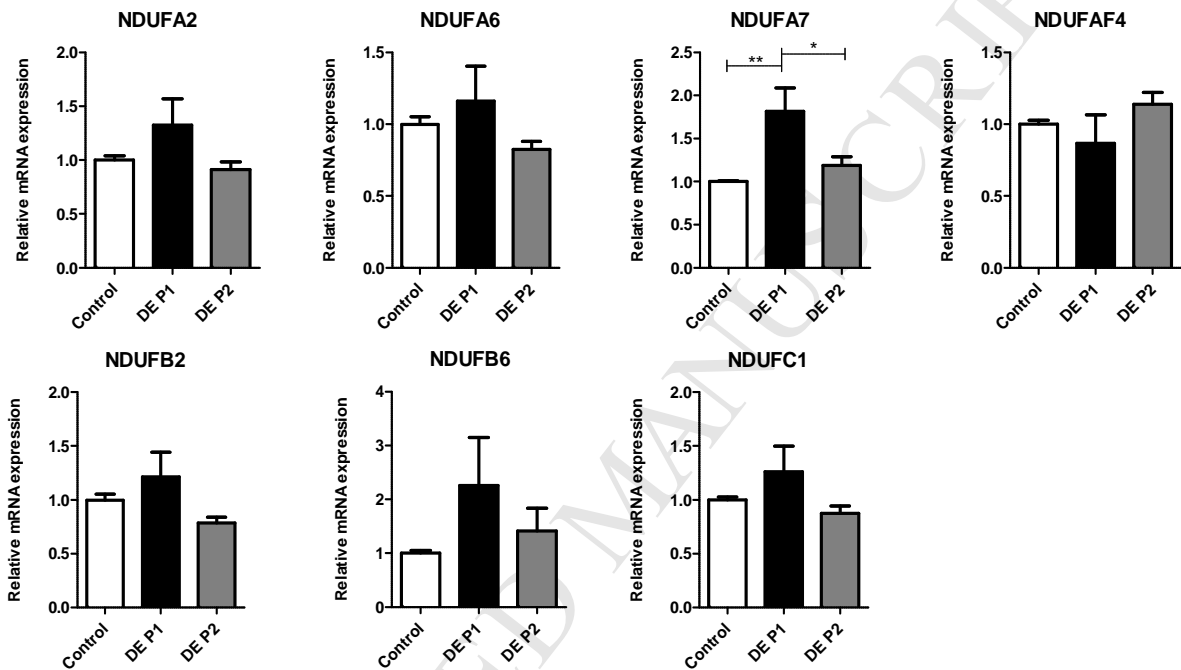
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332 **Figure 5:** Expression analysis of genes coding for the mitochondrial complex I. mRNA levels
 333 were determined by RT-qPCR. Data are expressed relatively to mRNA levels found in the
 334 Control group exposed to air and arbitrarily set at the value of 1. N=6, Means \pm SEM. *p
 335 <0.05 and ** p <0.01.

336

337 **4. DISCUSSION**

338 In this study, we compared cardiac effects after exposure with exhaust collected downstream
339 (P2) or upstream (P1) of a DPF using a standard diesel and engine, representative of the
340 current fleet and used under dynamic conditions to mimic emissions produced in urban
341 driving. In such conditions, we provide evidence that these DE produce a sustained cardiac
342 and mitochondrial dysfunction associated with an oxidative stress-independent impairment of
343 the mitochondrial function after 3 weeks of exposure in healthy rats.

344 Many conditions may affect diesel vehicle emissions, such as driving conditions, post-
345 treatment technologies that reduce regulatory pollutant emissions, or fuel composition. In this
346 study, the urban cold start driving condition produces the highest emissions of total gaseous
347 hydrocarbons and CO, whereas these emissions are below the limit for detection during the
348 other driving conditions, due to the catalysis. However, oxidation catalysis causes an increase
349 in NO₂ emissions, observed during the driving conditions. These observations are in
350 agreement with Martinet et al (2017) (Martinet et al., 2017) and these elevated NO₂ emissions
351 may contribute to the biological effects of DE, as previously suggested (Karthikeyan et al.,
352 2013). Note that the NO₂ levels in our study are similar between P1 and P2 due to the diesel
353 oxidation catalyst located upstream to regenerate the DPF with NO₂, an active oxidation agent
354 used in the regeneration process of the soot accumulated in the DPF (Kandylas and Koltsakis,
355 2002). Particle filtration significantly reduces particle mass emission, with a near 100%
356 efficiency associated to a decrease in alkane and PAH levels, as well as VOCs such as BTEX.
357 However, carbonyl compounds, acetaldehyde and formaldehyde, emissions remain abundant
358 and the levels increase after the DPF. These results indicate that the particle trap may have
359 changed the composition of exhaust, but additional analyses are required to confirm it, even
360 though a previous study led to similar observations (Ratcliff et al., 2010).

361 In these experimental conditions, with or without DPF, our results suggest that repeated DE
362 exposures result in cardiac dysfunction. Indeed, the significant increases in LV diastolic and
363 systolic diameters observed, show LV dilatation associated with reduced LV contractility, but
364 LV dysfunction is only moderate since cardiac output is not modified. These results indicate
365 that LV remodeling is sufficient to maintain global cardiac function. However, it should be
366 outlined that our experimental conditions probably underestimate the deleterious effects of
367 repeated DE exposure. Indeed, LV function was only determined 16-18 h after the last
368 exposure, excluding the deleterious direct effects of DE, as observed 1 h after acute DE
369 exposure. Furthermore, it is tempting to state that if LV function had been measured 1 h after
370 the last exposure of the repeated DE exposure, LV dysfunction would have been more
371 marked, since it would combine both acute direct as well as chronic adaptive mechanisms.
372 Similarly, one might question whether longer repeated DE exposure would have induced
373 more marked cardiac dysfunction.

374 Moreover, our results clearly show the importance of gaseous phase vs. particles since both
375 P1, i.e. whole exhaust, and P2, i.e. gaseous phase without particles, induce a similar increases
376 in LV diastolic/systolic diameters after either single or repeated DE exposure. Consequently,
377 our study expands the knowledge of DE's deleterious effects on health, since, as shown
378 previously, DE particles alone decrease fractional shortening and induce end-diastolic
379 diameters after repeated exposures in healthy rats (Bradley et al., 2013).

380 The mechanisms underlying the cardiovascular effects of DE remain only partially
381 understood, but a systemic inflammatory response associated with an oxidative stress initiated
382 in the lung may relate to the triggering of the cardiovascular effects (Brook et al., 2010;
383 Nemmar et al., 2018). Our laboratory previously observed a limited accumulation of oxidative
384 damage in the lungs following repeated exposure to DE (Douki et al., 2018). Consistent with
385 these previous findings, our data provide no evidence of a cardiac oxidative stress, as cardiac

386 redox parameters remain unchanged. Indeed, neither cardiac antioxidant enzymes, nor
387 reduced/oxidized glutathione ratio, were significantly modified after acute and repeated
388 exposures. We also measured activity of aconitase, a sensitive mitochondrial enzyme of
389 superoxide (Gardner, 2002), but observed no differences. Even though we cannot rule out a
390 brief effect of reactive oxygen species, oxidative stress does not seem to be a major triggering
391 event for the cardiovascular effects observed in this study. DE-related oxidative stress is often
392 attributable to PM and related to their physico-chemical characteristics, in particular surface
393 bound organic compounds such as metals, quinones and PAH (Charrier and Anastasio, 2012;
394 Cho et al., 2005; Crobeddu et al., 2017). The concentration of these particulate constituents
395 evaluated in this study is lower, as previously evaluated in new diesel engines than in
396 traditional DE (McClellan et al., 2012); this may explain the lack of a sustained oxidative
397 stress in this study.

398 To identify the cellular mechanisms contributing to cardiac dysfunction following DE
399 exposures, we next focused on mitochondrial function, the primary source of energy in the
400 myocardium. For this purpose, we first evaluated oxygen consumption in situ, in saponin-
401 skinned cardiac fibers that ensure global mitochondrial function assessment in intact
402 mitochondria (Veksler et al., 1987). We showed that respiration rate with complex I-linked
403 substrates (glutamate and malate) was affected in the hearts of rats exposed to repeated
404 filtered DE. However, when succinate was provided as substrate, mitochondrial respiration
405 was not affected, suggesting that the electron chain was not affected downstream of the
406 complex II. As we did not observe any mitochondrial dysfunction after an acute exposure, we
407 next performed further mitochondrial investigations in the hearts of rats exposed to repeated
408 DE and observed a decrease in complex I activity specifically. Indeed, the tissue activities of
409 complexes III and IV, and citrate synthase were not modified. Taken together, these results
410 indicated that decreased respiration rates with glutamate and malate are due to decrease in

411 complex I function. Complex I dysfunction has also been observed in several diseases
412 including heart failure (Scheubel et al., 2002), ischemia-reperfusion (Kang et al., 2018), or
413 during chronic cardiac pressure overload (Schrepper et al., 2012). DE can instigate adverse
414 cardiovascular response by activation of the sympathetic nervous system mediated through
415 activation of pulmonary sensory receptors and adrenergic receptors (Robertson et al., 2014).
416 This activation is associated with elevated blood pressure and increases vulnerability to
417 ischemia and reperfusion injury (Robertson et al., 2014). Although the underlying molecular
418 mechanisms connecting cardiac mitochondrial complex I defect and pulmonary exposure to
419 DE remain to be deciphered, this mitochondrial dysfunction could participate, at least
420 partially, in this vulnerability after DE exposure.

421 The impact on mitochondria of repeated exposure to DE was further investigated by
422 transcriptional analysis of LV tissue. Several pathways relating to mitochondria were
423 identified in LV tissue from exposed rats, such as “oxidative phosphorylation” and
424 “mitochondrial dysfunction” (P1 vs. Control) or “swelling of mitochondria” (P2 vs. Control),
425 indicating the impact of the DE exposures on the mitochondria. Individual gene expression
426 analysis revealed that very few genes were significantly regulated by DE exposure, probably
427 due to an adaptive response after exposures, since these results were obtained after 3 weeks of
428 repeated exposures, with 16 h post-exposure. In these conditions, our results showed that
429 NDUFA7 mRNA expression was slightly but significantly up-regulated in rats exposed to DE
430 and a similar trend was observed for NDUF6. These genes encode subunits of
431 NADH:ubiquinone oxidoreductase (complex I) and this result indicates that the changes in
432 complex I activity may not be due to a decrease in gene expression but may reflect an
433 adaptive mechanism in response to the decrease in complex I activity. Another regulatory
434 pattern for changes in respiratory complex activities may be considered such as impaired

435 assembly of subunits to supercomplexes (Rosca et al., 2008) but further investigations are
436 needed.

437 Complex I is the largest multi-subunit complex of the respiratory chain and is one of the
438 complexes that generate the proton-motive force required for ATP synthesis. In order to
439 evaluate the consequences on the synthesis of ATP, we evaluated ATP production in two
440 isolated mitochondrial subpopulations, subsarcolemmal (SSM) and interfibrillar mitochondria
441 (IFM), and observed a decrease in ATP synthesis capacity selectively in IFM. As described in
442 the literature, subpopulations of mitochondria are structurally and metabolically distinct and
443 are differently susceptible to pathological stimuli (Hollander et al., 2014). The defect in
444 mitochondrial ATP synthesis observed in the IFM population is also consistent with the
445 known susceptibility of these mitochondrial subpopulations to heart failure (Schwarzer et al.,
446 2013) or aging (Hofer et al., 2009). This result is clinically relevant as the heart is particularly
447 vulnerable to limited ATP supply because of its large energy request.

448 It is noteworthy that these mitochondrial changes are observed with whole DE (P1, mRNA
449 expression), filtered DE (P2, oxygen consumption), or both (ATP synthesis and complex I
450 activity). These apparent discrepancies could be explained by methodological differences.
451 Within the respiratory chain, O_2 consumption measured from LV skinned cardiac fibers
452 makes it possible to maintain the interactions of assembled complexes and electron
453 transporters, which influence the measurement of respiratory capacity, whereas complex
454 activity as well as ATP synthesis capacity are independent of these influences. These results
455 might also translate the relative effect of the particle and gas phases and their different
456 interaction with the mitochondria. Whole DE contains respirable soot-particles, but although
457 the cardiovascular effects of particles have been extensively explored, few studies attempted
458 to estimate the mitochondrial function in the heart. A previous study performed in mice
459 showed a time-dependent and reversible decrease in O_2 consumption after acute residual oil

460 fly ash (ROFA) (Marchini et al., 2013). Though restricted to an acute ROFA exposure, this
461 result showed an impairment of mitochondrial function associated with deficient cardiac
462 contractility. With regard to gaseous phase, some combustion-related gases, such as CO
463 (Reboul et al., 2017) and SO₂ (Qin et al., 2016), have previously shown to induce
464 mitochondrial effects. However, it remains difficult to connect an effect in response to a
465 specific pollutant in a complex mixture, for which the composition varies according to the
466 driving conditions and after treatment. Notwithstanding these limitations, the findings of the
467 present study are new and suggest that the mitochondrial impairment contributes to the
468 clinical cardiovascular events observed after DE exposure.

469 In conclusion, this study performed with diluted DE emitted from an engine used under
470 dynamic conditions revealed sustained cardiovascular and mitochondrial effects attributable
471 to the gas phase, possibly aldehydes and/or NO₂, but further study is warranted to clarify the
472 precise role of these pollutants. Although the predictivity of these results to humans remains
473 delicate due to limitations inherent in rodent studies, the effects observed in cardiac
474 mitochondria may suggest significant consequences in terms of cardiac effects for vulnerable
475 populations with underlying energy deficit such as patients with heart failure or the elderly.

476 **Conflict of interest statement**

477 Authors declare that there are no conflicts of interest.

478 **Funding**

479 This work was supported by ADEME (French Environment and Energy Management
480 Agency; Cardiox project, grant number 2013-1-237), Inserm (grant number ENV201207) and
481 the Regional Council of Haute-Normandie.

482 **Acknowledgement**

483 The authors would like to thank P. Scotti di Vettimo and Dr N. Theret for their advice in
484 bioinformatics analysis.

ACCEPTED MANUSCRIPT

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