



Comparative study on gene expression profile in rat lung after repeated exposure to diesel and biodiesel exhausts upstream and downstream of a particle filter

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Manuscript Details

Manuscript number Title ENVPOL_2020_2800_R1
Comparative study on gene expression profile in rat lung after repeated exposure to diesel and biodiesel exhausts upstream and downstream of a particle filter

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Abstract

Biodiesel is considered as a valuable and less toxic alternative to diesel. However, cellular and molecular effects of repeated exposure to biodiesel emissions from a recent engine equipped with a diesel particle filter (DPF) remain to be characterized. To gain insights about this point, the lung transcriptional signatures were analyzed for rats (n=6 per group) exposed to filtered air, 30% rapeseed biodiesel (B30) blend or reference diesel (RF0), upstream and downstream a DPF, for 3 weeks (3h/day, 5 days/week). Genomic analysis revealed a modest regulation of gene expression level (lower than a 2-fold) by both fuels and a higher number of genes regulated downstream the DPF than upstream, in response to either RF0 or to B30 exhaust emissions. The presence of DPF was found to notably impact the lung gene signature of rats exposed to B30. The number of genes regulated in common by both fuels was low, which is likely due to differences in concentrations of regulated pollutants in exhausts, notably for compound organic volatiles, polycyclic aromatic hydrocarbons, NO or NOx. Nevertheless, we have identified some pathways that were activated for both exhaust emissions, such as integrin-, IGF-1- and Rac-signaling pathways, likely reflecting the effects of gas phase products. By contrast, some canonical pathways relative to "oxidative phosphorylation" and "mitochondrial dysfunction" appear as specific to B30 exhaust emission; the repression of transcripts of mitochondrial respiratory chain in lung of rats exposed to B30 downstream of DPF supports the perturbation of mitochondria function. This study done with a recent diesel engine (compliant with the European IV emission standard) and commercially-available fuels reveals that the diesel blend composition and the presence of an after treatment system may modify lung gene signature of rats repeatedly exposed to exhaust emissions, however in a rather modest manner.

Keywords Biodiesel; diesel exhaust; lung; particle filter; transcriptome analysis

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Accepted manuscript

Dear Editor,

Please find here attached the revised version of our manuscript (ENVPOL_2020_2800) entitled "Comparative study on gene expression profile in rat lung after repeated exposure to diesel and biodiesel exhausts upstream and downstream of a particle filter" by Lecureur et al.

Thank you for the opportunity to address the reviewers' concerns. Please find attached our revised manuscript for consideration of publication in *Environmental Pollution*. We have addressed every issue highlighted by the reviewers. We have also totally reshaped the abstract.

The specific answers to reviewers are attached.

Once again, thank you for considering our revised manuscript,

Yours sincerely,

Valérie Lecureur,

Corresponding author for Ms. ENVPOL_2020_2800

Responses to Reviewer 1:

We thank the reviewer for his supportive comments and helpful suggestions. We have included here our responses:

1. According to data from Table 1, these results present heterogeneity (e.g. for PAHs in RFO: P1 measure 1 = 1.02 and P1 measure 2 = 9.89). What was the reason of this heterogeneity ?

As we indicated in the first version of the manuscript, we observed “heterogeneity of PAH concentrations”. Such heterogeneity concerns essentially the measure of naphthalene, the most represented PAHs that we find especially low in the first measure of the RFO campaign; it is not easy to explain these differences in naphthalene concentrations between measures. In the reviewed version, we now added the following sentence: “In fact, a reduced concentration was observed for PAHs except for naphthalene which represents more than 80% of the measured PAHs. The heterogeneity of the PAH concentration upstream DPF may be due to a lower concentration of naphthalene during the first campaign measure, which remains to explain. (Page 7, lines 206-209).

2. “According to data from table 1, BTEX, aldehyde, alkane and PAHs concentrations were strongly reduced in exhausts downstream of DPF (P2) for B30 fuel, as compared with corresponding concentrations in exhausts downstream of DPF (P2) for RFO fuel. However, the number of genes regulated by B30 fuel after DPF (781 genes) was 1.8 fold higher than that regulated by RFO fuel (426 genes). The authors should explain/discuss this issue”.

It is true that the number of differentially regulated genes is higher in lung of rats exposed to B30 than those exposed to standard diesel RFO (in P2) (781 versus 426 genes) whereas aldehydes, alkanes and PAHs concentrations were found lower in B30 exhaust than RFO exhaust. However, BTEX, NO and NO_x concentrations were found higher in B30 than in RFO exhausts downstream DPF (See new data in the Table S1) and such differences may at least in part explain this higher number of genes differentially regulated by B30 exhausts. In the revised manuscript, we now added the following sentence: “So, the greater number of regulated genes in lung of rat exposed to B30 compared to those exposed to RFO may be related at least in part to the higher concentration of fluoranthene, pyrene, NO, NO_x and VOCs such as BTEX, especially of xylene.” (page 15, lines 445-448).

3. “According to data from table 1, BTEX, aldehyde, alkane and PAHs concentrations were strongly reduced in exhausts downstream of DPF (P2) for B30 fuel. However, the number of genes regulated by B30 in P2 (downstream DPF) was 5.1 fold higher than in P1 (upstream DPF) (781 versus 153 genes). The authors should explain/discuss this issue”.

It is true that the number of differentially regulated genes is higher downstream DPF (in P2) than upstream (in P1) (781 versus 153 genes) whereas whole diesel exhaust in P1 contains particles and thus represents a more complex mixture than those in P2, limited to gas phase. The same observation was also done when rats were exposed to RFO emissions. Such data were surprising because we have previously observed in a transcriptomic analysis done on heart tissue of the same rats exposed to diesel emissions that the number of genes was lower downstream DPF (Karoui et al, 2019). In the revised manuscript, we added the following sentence: “However, despite such decreases of exhaust concentrations, the number of regulated genes by B30 emissions was higher downstream than upstream the DPF. Such data were rather surprising since an opposite situation was previously observed in cardiac tissue of rats exposed to diesel emissions (Karoui et al, 2019), suggesting some specific tissue response to gas phase of exhaust or to remaining ultrafine PM.” (Page 13, 401-405).

“The authors reported that aldehyde concentrations (mostly formaldehyde and acetaldehyde) were highly increased after DPF (P2) for RFO, but were decreased for B30 blend. The authors should explain/discuss this issue”.

It will be interesting to know why aldehyde and especially acetaldehyde and formaldehyde contents increase in RF0-P2, whereas they decrease in B30-P2/ However, this was not the primary purpose of this study and we can only describe these results, which could explain at least in part the difference in the effects of both fuel exhausts observed in P2, without particles. In accordance with the literature, B30 generates slightly less total aldehyde emission than diesel does. Moreover, when oxidation reaction occurs in catalyst, the intermediate products such as formaldehyde are formed. It is possible that less formaldehyde in B30 emissions was produced because of the 30% dilution of exhaust. In the revised manuscript, we added the following sentence: “As formaldehyde may be an intermediate product from other aldehydes or hydrocarbons during oxidation reaction in catalyst, its decrease in exhausts from B30 in comparison to those of RF0 may be related to the 30% dilution of diesel and thus to the lowest concentration of such initial compounds in B30 blend.” (page 13, lines 395-400).

Responses to Reviewer 2:

We thank the reviewer for his supportive comments and helpful suggestions. We have included here our responses:

1. “The abstract is quite confusing and it would be best if the authors first described what transcription pathways are induced by the whole exhaust fuels compared to the air controls and then highlight differences or new pathways attributed to the filtered emissions. The concentration of exhaust that the animals were exposed to for each fuel would be good to include in the abstract”.

As suggested by reviewer 2 and 4, we reshaped the abstract.

However, we did not first described what transcription pathways are induced by the whole exhaust fuels compared to the air controls and then highlight differences or new pathways attributed to the filtered emissions, because our data were essentially focused on B30 exhausts and because we did not compared the transcription pathways downstream and upstream the DPF for RF0.

In this new abstract, we also gave now some informations concerning methods and exposure procedure. In addition, as suggested by the Reviewer 4, we now better overall summarize the results.

2. “At the end of the discussion the Douki et al paper is introduced as being part of this overall program and perhaps has broader effects that this genomic analysis is trying to explain. It would be good to summarize this paper in the introduction as a launch point for this current study.”

As suggested by the Reviewer, we now introduce Douki et al article with the other one (Karoui et al, 2019) from our research consortium in the Introduction section. In the revised manuscript (Page 4, lines 95-99), we have thus indicated: “Concerning our studies, we evaluated the global genomic effects of repeated exposure to diesel exhaust on rat heart and identified some pathways related to mitochondrial dysfunction (Karoui et al, 2019). In lung tissue of rats exposed to diesel and biodiesel exhausts, no significant alteration of pathways related to genotoxicity was additionally observed (Douki et al, 2018).

3. “The schematic of the exposure system and experimental design does not include air controls and that would be more complete so that people understand this was a balanced experimental design”.

We thank the Reviewer for this comment. We now add on the graphical abstract the exposure to air (as control).

4. “The abstract says that the exposures caused “modest adverse effects”. What exactly are these “adverse effects”. I see some changes in genomic expression but don’t know if they are adverse”.

It is true that the transcriptomic analysis alone cannot permit to know if the effects of exposure to diesel exhaust can cause adverse effects. That is why, and under the recommendation of one of the reviewers, we

now added new data based on histological analyses in the revised version of this article. In the Figure S2, we showed the histological scores of lung inflammation, (that we can consider as adverse effects), which appear slightly higher in B30-exposed rats than in RFO-exposed rats both upstream and downstream of DPF. Such data are now presented in the results section (page 8) and discussed page 13.

5. Also there is no data on the levels of accompanying gases (Nox, CO etc.) This may be previously published but the readership would be interested in a standard summary table of this.

In fact, some of these data were previously published in Douki et al. (2018). As requested, we added data on regulated gaseous pollutants concentrations such as NO, NOx, CO and total gaseous hydrocarbons in a supplementary table (Table S1) and we now mention some details concerning operating conditions (NEDC cycle). The following new paragraph appears now in the revised version of the article: "The exhaust characterization was performed upstream (P1) and downstream (P2) of the DPF during the NEDC cycles. The results showed that DPF reduced the total PM concentrations for both fuels (Table S1). NO and NOx concentrations were found higher in B30 than RFO exhausts, in contrast to CO concentrations, and the presence of DPF did not change the total gaseous hydrocarbon concentrations (Table S1)." (Page 7, lines 194-

6. "The figure legends do not say how many animals for each group were subjected to the genomic analysis. I assume it was all 6 but it is not clear."

As recommended by the Reviewer, we now added the number of rats in each group on the Figure 1. The number of rat lung tissues used for genomic analysis was precise at Page 6, line 165: "Raw affimetrix data (.CEL files) from 36 arrays (corresponding to six lung samples from two campaign of exposure to RFO and B30 with 6 rats/group)..."

7. "This analysis was performed on a small piece of lung (which comprises 40 different cell types). The origin of the genomic changes (whether this is resident cells or perhaps new cells) should be discussed."

We thanks the Reviewer for this pertinent suggestion. In fact, the genomic changes may be related to the response of resident but also to the recruited cells in the lung tissue. We therefore propose the following sentence: "Because of such mild lung inflammation, we cannot argue that the genomic signatures reflect only the molecular changes within lung resident cell population, but also probably those of infiltrated cells." (page 13, lines 385-387).

8. "Were mass measurements done after the filter to ensure the filter actually worked and to what extent? "

As suggested by the Reviewer, we now added the data on PM mass measurement in a supplementary table (Table S1). These data are now included in the text as followed: "The results showed that DPF reduced the total PM concentrations both for both fuels (Table S1)" (Page 7, lines 195-196) and "The efficiency of the DPF was confirmed by the near absence of PM downstream the DPF" (Page 13, line 388).

9. "Terming the filter a "depollution device is a little strange."

We have changed device by system

10. "The Finch et al paper if I recall did not study genomic responses to biodiesel and therefore the statement on page 7 line 92 is misleading.

We agree with the Reviewer that in Finch et al article, which was one of the first to study a subchronic exposure of rats to emission from a diesel engine burning soybean for a long period (13 weeks), no genomic study was done. Therefore, we removed this reference from the paragraph concerning transcriptomic approaches in the introduction.

11. “The Stevens et al paper that was cited also described genomic pathway analyses for several types of diesel samples and parallels where appropriate between the current study and earlier work would be informative.”

We now discuss about Stevens et al data in the text as followed: “Also, the identification of the “oxidative phosphorylation” pathway in mice exposed to diesel exhaust (Stevens et al., 2008)” (Page 14, lines 429-430).

Responses to Reviewer 3:

We thank the Reviewer for his supportive comments and helpful suggestions. We have included here our responses:

1. “Details of engine operation are necessary to be included. Is the engine operating with or without load?. If it was under a load, what is the operational cycle? Such aspects are important, since the operation mode of the engine may alter the emission composition”

We thank the Reviewer for this comment. As the exposure procedure was already published in the Douki et al, article, we limited the detail of engine operation in this article. But, as requested, we now add these information in the text: “The diesel engine was placed in a test bench cell equipped with a dynamic asynchronous chassis dyno, which allows continuous control of both engine speed and load, and was used under dynamic conditions according to the “New European Driving Cycle” (NEDC).” (page 4 , lines 111-113).

2. “Authors fully acknowledge that some integrative toxicologic endpoint, such as cell counts obtained by bronchoalveolar lavage, could be of use. However, they had lung tissue material. Did they measured quantitative markers of pulmonary alteration, such as phenotypic changes of airways (lowe and upper), including mucin profile secretion or tissular accumulation of inflammatory cells? Do they have samples of blood, bone marrow or other organs? Although I fully acknowledge that the emphasys of the study was on the different gene expression profiles, perhaps these additional pieces of information could provide a supportive background for the comparative toxicity of both fuels”

We thank the Reviewer for these comments. We do not have no more lung tissue to realize new measurements such as mucin profile secretion for example. We have previously counted blood samples and as already indicated in the manuscript, we did not see any difference between groups of rats (page 8, lines 231-232). It is true that we have some tissue samples from others tissues (heart, liver and kidney); the analysis of diesel exhaust on heart tissue has been the subject of a publication (Karoui et al, 2019) but the analysis of other tissue samples was not done yet and is not the subject of this article.

As suggested by the Reviewer, we now presented some lung histological data such as histological score of lung inflammation and some lung tissue section showing alveolar macrophages in the Figure S2.

These data are now presented in the text as followed: “Histopathological examinations did not show major structural lung tissue alteration. It however revealed the presence of pigmented macrophages in the alveolar space of rats exposed to exhausts in P1, but not in the group of rats exposed to filtered air nor in the group of rats exposed to both fuels downstream the DPF (Figure S2). The histological scores of lung inflammation showed no or a mild inflammation in the lung of rats exposed to filtered air; they were higher in B30-exposed rats than those of rats exposed to RF0 both downstream the DPF (100% of mild-moderate inflammation versus 44%) and upstream the DPF (83% of mild-moderate inflammation versus 67%) (Figure S2).” (Page 8, lines 232-240) and in the discussion section of the article Page 13, lines 381-387.

3. “Finally, I think that conclusions should be somehow made more simple, since the general reader of Environmental Pollution may be not used to the jargon of molecular toxicology.”

As suggested by the Reviewer, we re-worded the conclusion to simplify it; see page 16, lines 481-485.

Responses to Reviewer 4:

We thank the Reviewer for his supportive comments and helpful suggestions. We have included here our responses:

1. Abstract: a better overall summary of the results should be provided

As suggested by Reviewer 2 and Reviewer 4, we reshaped the abstract. In this revised abstract, we also give now some information concerning methods and exposure procedure. And, as suggested by the reviewer4, we now better overall summarize the results.

2. Results, section 3.2 – more appropriate title should be used.

We have changed the title of this section and replace “Measurement of different parameters in animals” by “Analysis of tissue and of biological parameters”.

3. Results, section 3.2, line 212 – the weight changes between the groups are not significant (except of an RF0/P1 vs. controls comparison). This should be clearly indicated in the text.

We have changed the previous sentence : “Overall, the weight-gain of rats exposed to fuels was lower than those exposed to air, but rats exposed to RF0 before DPF (P1) showed a significant reduction in weight-gain when compared to their respective filtered air controls” by a new sentence : “Overall, there were no weight change between groups, except in the group of rats exposed to RF0 before DPF (P1), which showed a significant reduction in weight-gain when compared to their respective filtered air controls (Table S2).” (Page 8, lines 229-231).

4. Results, section 3.3 – correct a typo “Genes differentially expressed...”

The “Gene differentially expressed...” was replaced by “Genes_s differentially expressed”

5. “Results, section 3.4, line 306 – “oxidative phosphorylation”

“oxydative phosphorylation” was changed for “oxidative phosphorylation”.

6. “Discussion, line 354 (point 3) – it is not clear for which groups the comparison is being made”

Thanks for this comment. To avoid confusion, we removed the previous sentence: “The same procedure was applied to the canonical pathways found for B30-exposed rats in P1 but no z-score was attributed to them.” to only present data where z-score were attributed to pathways, i.e. B30-P2 (Page 12, lines 353-354).

7. “Table 1 – big differences between measurements 1 and 2 for some compounds were found (e.g. BTEX, B30). Some explanation should be provided.”

We thank the Reviewer for its comment, which was also pointed out by the first reviewer. It is true that we found some heterogeneity between the 2 measurements done for each exposure campaign. Concerning BTEX measurement, we found an higher concentration of xylene in the second measurement in comparison to the first which explain the highest value of BTEX in emissions of B30 upstream the DPF. This detail is now precise in the text as followed:“The heterogeneity in BTEX concentrations measured in B30 emissions upstream the DFP was due to a highest xylene concentration in the second measure than the first.” (Page 8, lines 217-218).

8. The authors should discuss the reasons for more pronounced response after P2 exposures, particularly for B30.

We thank the Reviewer for its comment, which was also pointed out by the first reviewer.

Please find here our responses for both reviewers : It is true that the number of differentially regulated genes is higher in lung of rats exposed to B30 than those exposed to standard diesel RF0 (in P2) (781 versus 426 genes) whereas aldehydes, alkanes and PAHs concentrations were found lower in B30 exhaust than RF0 exhaust. However, BTEX, NO and NOx concentrations (new data in the Table S1) were found higher in B30 than in RF0 exhausts downstream DPF and such differences may at least in part explain these higher number of genes differentially regulated by B30 exhausts.

In the revised manuscript, we now added the following sentence: "So, the greater number of regulated genes in lung of rat exposed to B30 compared to those exposed to RF0 may be related at least in part to the higher concentrations of fluoranthene, pyrene, NO, Nox and VOCs such as BTEX, especially of xylene." (page 15, lines 445-448).

9. For gene expression analysis, microarray technology was used, although NGS provides more comprehensive results. The authors should explain why they used this approach and mention it in the study limitations.

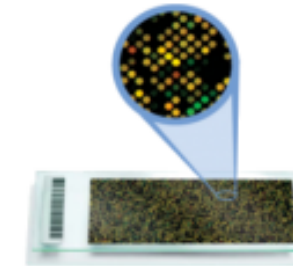
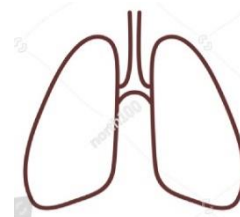
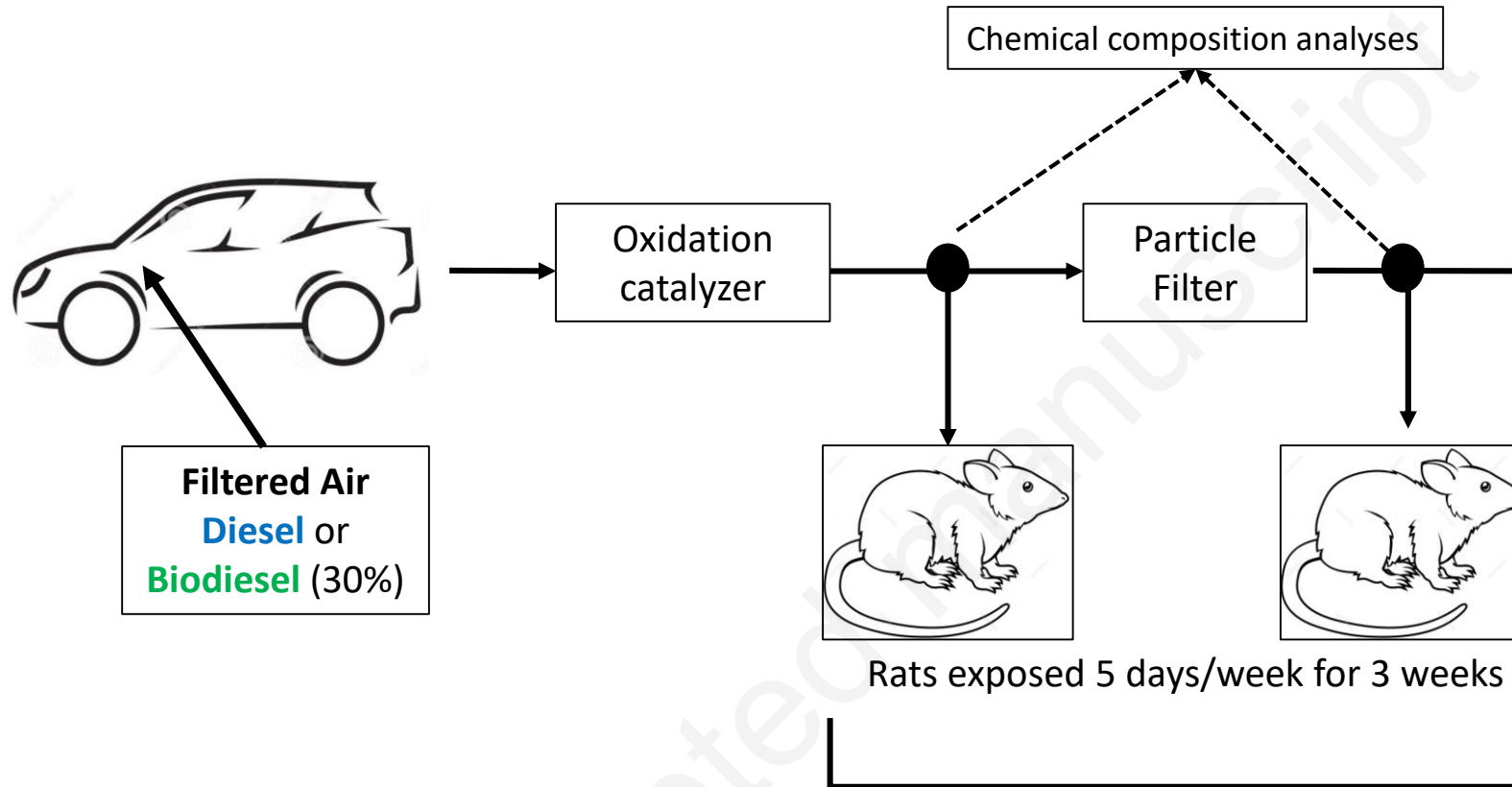
When we started this collaborative project, the next-generation sequencing was not so widespread and anyone in the consortium team wasn't familiar with this technology. I think that if we would start this type of study in 2020, we will use NGS. Therefore, we propose the following sentence in the "limitations" paragraph: "Second, this transcriptomic study could have been carried with a more recent and more efficient approaches, such as the next-generation sequencing (NGS), but when this study began, NGS was quite expensive and not so spread." (Page 16, lines 472-474).

10. How do the exposure doses compare to real-life exposure scenarios?

Some comments on the realistic exposure were previously published in the article of Douki et al, 2019. But as suggested by the Reviewer, we decided to add some sentences concerning the real-life scenario of exposure in this article in the Materials and Methods part. Therefore, we now added the following sentence: "This design of exposure is representative of a realistic exposure because the dose was based on the quantile 75% value of the concentrations found inside a car in an urban traffic (Morin et al, 2009). And the daily 3 h exposure corresponds to a high but plausible exposure duration, to which people may be exposed on their 5 working days" (Page 5, lines 136-140).

Highlights:

- Repeated lung exposures to diesel or biodiesel induce low level expression of genes
- Exposure to diesel or biodiesel induces a different profile of gene expression
- Diesel particle filter modifies exhaust composition and gene expression profile
- Mitochondrial dysfunction is observed downstream the diesel particle filter



Comparative studies of lung gene expression profiles

Comparative study on gene expression profile in rat lung after repeated exposure to diesel and biodiesel exhausts upstream and downstream of a particle filter

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Abstract

Biodiesel is considered as a valuable and less toxic alternative to diesel. However, cellular and molecular effects of repeated exposure to biodiesel emissions from a recent engine equipped with a diesel particle filter (DPF) remain to be characterized. To gain insights about this point, the lung transcriptional signatures were analyzed for rats (n=6 per group) exposed to filtered air, 30% rapeseed biodiesel (B30) blend or reference diesel (RF0), upstream and downstream a DPF, for 3 weeks (3h/day, 5 days/week).

Genomic analysis revealed a modest regulation of gene expression level (lower than a 2-fold) by both fuels and a higher number of genes regulated downstream the DPF than upstream, in response to either RF0 or to B30 exhaust emissions. The presence of DPF was found to notably impact the lung gene signature of rats exposed to B30. The number of genes regulated in common by both fuels was low, which is likely due to differences in concentrations of regulated pollutants in exhausts, notably for compound organic volatiles, polycyclic aromatic hydrocarbons, NO or NO_x. Nevertheless, we have identified some pathways that were activated for both exhaust emissions, such as integrin-, IGF-1- and Rac-signaling pathways, likely reflecting the effects of gas phase products. By contrast, some canonical pathways relative to "oxidative phosphorylation" and "mitochondrial dysfunction" appear as specific to B30 exhaust emission; the repression of transcripts of mitochondrial respiratory chain in lung of rats exposed to B30 downstream of DPF supports the perturbation of mitochondria function.

This study done with a recent diesel engine (compliant with the European IV emission standard) and commercially-available fuels reveals that the diesel blend composition and the presence of an after treatment system may modify lung gene signature of rats repeatedly exposed to exhaust emissions, however in a rather modest manner.

Keywords: Biodiesel; diesel exhaust; lung; particle filter; transcriptome analysis

Capsule: The lung gene signatures of rat exposed repeatedly to diesel and biodiesel vary with the diesel composition, the presence of a diesel particle filter and support modest lung adverse effects.

1. Introduction

According to global burden of diseases, ambient particulate matter (PM) pollution is the first environmental or occupational risk of deaths (Cohen et al., 2017). It causes an estimated 5.25% of all deaths and is the eighth-ranking global mortality risk factor in 2017 (GBD 2016 Risk Factors Collaborators, 2017). Increased mortality and morbidity related to ambient PM exposure is notably related to cardio-pulmonary diseases (Cohen et al., 2017; Miller et al., 2012; Pope et al., 2002). Road traffic, which is the third major source of the airborne particles (EEA Report 2019), and exposure to diesel exhaust particle (DEP), including fine particles such as PM_{2.5}, contribute to lung adverse effects, notably to exacerbations of asthma and to allergic airway diseases (Ghio et al., 2012; Steiner et al., 2016).

The diesel emissions of vehicles contain numerous chemical compounds, which are emitted in both the gaseous and particulate phases of the exhaust resulting from an incomplete combustion of diesel fuel. The main components of diesel exhausts comprise carbon monoxide and dioxide, nitrogen and sulphur oxides, volatile polycyclic aromatic hydrocarbons (PAHs) and PM ranging from fine (2.5-0.1 μm diameter) to ultrafine (<0.1 μm diameter), known to reach the deep airways. Toxic effects of these DEPs are related to their capacity to interact with alveolar macrophages and lung epithelial cells, triggering inflammation, oxidative stress and immune response (Ristovski et al., 2012), or to their direct translocation into the circulation (Nemmar et al., 2002, 2001).

Trans-esterified lipids of soybean and rapeseed oils have a potential for use in energy combustion. They represent alternative fuels when they are mixed at levels of 20-30% with standard diesel fuel, leading to some difference between biodiesel- and diesel-emissions (Bünger et al., 2012). The increased use of these biofuels, which accounted for about 7 percent of the energy use in transport in 2018 in Europe (EU Biofuels Annual 2019), may change atmospheric pollution profile. This potentially causes different health effects. Some depollution systems such as diesel particulate filter (DPF) installed on all new vehicles in European Union (since the European emission standard Euro V), permit to reduce particle emission. However, to date, knowledge concerning the toxicological effects of the biodiesel emissions from recent engine in the presence of depollution system is still insufficient (Godri Pollitt et al., 2019; Weitekamp et al., 2020).

Recently, “omics” approaches have been applied extensively in the field of toxicology to identify new biomarkers and to unravel molecular mechanisms of toxicity. Transcriptomic

87 studies based on microarray analysis to assess biological risks of diesel exhaust exposure were
88 usually realized after an acute exposure to exhausts (Pettit et al., 2012; Srivastava et al., 2014;
89 Stevens et al., 2008; Yanagisawa et al., 2004), and more rarely after a probably more relevant
90 sub-chronic exposure (Maresh et al., 2011). A recent review pointed out the interest of 91
“omics” for investigating biodiesel-induced pulmonary toxicity (Selley et al., 2019). Indeed, 92
few studies have analyzed the genomic response of rodents exposed repeatedly to 93 diesel/
biodiesel exhausts, except those from the “FuelHealth project” and from our research 94
consortium. The authors of the “FuelHealth project” analyzed the genomic impact of a 28- 95
days inhaled diesel and biodiesel exhaust exposure on rat brain and lung tissues, but with 96
however only a limited number (32) of analyzed interest genes. Concerning our studies, we 97
evaluated the global genomic effects of repeated exposure to diesel exhaust in rat heart and 98
identified some targeted pathways related to mitochondrial dysfunction (Karoui et al., 2019). 99
In lung tissue of rats exposed to diesel and biodiesel exhausts, no significant alteration of
100 pathways related to genotoxicity was additionally observed (Douki et al., 2018).

101 The present study is a part of our consortium research project concerning diesel/biodiesel
102 exhaust effects and was designed to determine and compare the alterations of gene 103
expression in lung of rats exposed repeatedly to 30% rapeseed biofuel (B30) or reference 104
diesel (RF0), upstream and downstream of a DPF depollution system, through performing a 105
pan-genomic microarrays-based transcriptomic analysis.

106

107 **2. Materials and Methods**

108 *2.1. Exhaust generation and monitoring of emissions*

109 Diesel emission was produced by a light duty four cylinder direct-injection engine equipped
110 with an oxidation catalyst, with or without a wall-through DPF, as previously described
(Douki 111 et al., 2018). The diesel engine was placed in a test bench cell equipped with a
dynamic 112 asynchronous chassis dynamometer, which allows continuous control of both engine speed
and load; 113 it was used under dynamic conditions according to the “New European Driving
Cycle” (NEDC). 114 The standard fuel PSA4 (RF0) used was a low content sulfur fuel. It was
mixed to yield 30% 115 rapeseed methyl ester content (B30). Emissions from an internal
combustion engine 116 (European Euro IV standard diesel) were drawn directly from the
exhaust line. The primary 117 dilution by a factor 10 was performed by a Fine particle
sampler (Dekati Finland). Average

concentrations of regulated pollutants in RFO and B30 exhausts during NEDC cycling before dilution and exposure were previously published (Douki et al., 2018).

Emission components were measured from the atmosphere collected upstream the DPF, which consists in both gaseous and particulate phases, while that collected downstream contains the gaseous phase. The particulate phases were sampled upstream of the DPF by using a sampling system consisting in a transfer line heated according to the temperature of the emitted gases and heated filter. The gases were trapped using various adsorbing cartridges (e.g., Amberlite XAD-2 resin, multibed carbotrap (Carbosieve III, carbotrapB et C), 2,4-Dinitrophenylhydrazine) selective for the different types of targeted compounds. The sampling flow was controlled using mass flowmeters and a pump, as indicated in Fig. S1.

2.2. Animals housing and exposure

Rat exposure to exhaust has been previously described by our research consortium (Douki et al., 2018). Briefly, pathogen-free male Wistar rats weighing 270-300 g (Janvier Inc, Le Genest Saint Isle, France) were randomly divided into 6 groups, each containing 6 animals, and were placed in inhalation chambers (Anselme et al., 2007). Two exposure experiments were conducted with fuel exhaust: rats exposed 3 h/day x 5 days per week x 3 weeks to RFO or B30 were collected either at P1 (upstream DPF) or P2 (downstream DPF) (Fig. S1). In parallel, a control group was exposed to filtered air for each exposure campaign. This design of exposure is representative of a realistic exposure because (i) the dose was based on the quantile 75% value of the concentrations found inside a car in an urban traffic (Morin et al, 2009) and (ii) the daily 3 h exposure corresponds to a high but plausible exposure duration to which people may be exposed on their 5 working days. About 16 h after the last exposure, rats were sacrificed, blood samples were collected for cell counting and lungs were removed. All analyses were performed in a blind manner. Animal studies were reviewed and approved by the Committee on the Ethics of Animal Experiments under the authority of the French Ministry of Higher Education and Research (Authorization number 00291.01). They were carried out in strict accordance with the recommendations found in the Guide for the Care and Use of Laboratory Animals, EEC Council Directive 2010/63/EU.

2.3. Lung histology

A piece of lung (left upper lobe) was fixed with 4% formol, embedded in paraffin and sectioned at a thickness of 3 μ m on a microtome. Prepared sections were stained with hematoxylin-eosin-saffron (HES). Histopathological evaluation was performed by a certified pathologist. Sample identification was coded to ensure unbiased evaluation.

2.4. Microarray analysis

RNA from frozen lung tissue (piece of right lobe) was isolated using RNeasy Plus mini kit following manufacturer's protocol (Qiagen, Courtaboeuf, France). The RNA quantity and quality, assessed by a RNA integrity number superior to 9, were evaluated with a Nanodrop spectrophotometer and the Agilent 2100 Bioanalyser (Agilent technologies, Santa Clara, CA, USA), respectively. RNA was reverse-transcribed, converted to biotinylated complementary RNA and hybridized to GeneChip® RAGENE 2.0 ST Arrays, representing the whole rat genome, at the genomic platform of the Cochin Institute (Paris, France), according to Affimetrix protocol (Affimetrix, Santa Clara, CA, USA). Microarrays were washed and stained, and fluorescent images were obtained using the Affymetrix 3000 Scanner. Raw affimetrix data (.CEL files) from 36 arrays (corresponding to six lung samples from two campaign of exposure to RF0 and B30 with 6 rats/group) were transformed by the Robust Multichip Analysis method, corresponding to a correction, normalization and summarization of expression values. Standard quality metrics of Partek® and principle component analysis (PCA) allowed to detect potential outliers. The Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Mountain View, CA) was used to identify and associate signalling pathways and biological functions to the experimental transcriptomic results. Networks were "named" on the most prevalent functional group(s) present, whereas Canonical Pathway (CP) analysis identified function specific genes significantly present within the networks.

2.5. Quantitative real-time PCR

Total RNAs were reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Courtaboeuf, France). qPCR assays were next performed using the fluorescent dye SYBR Green methodology and an ABI 7900 detector (Applied Biosystem). The KiCqStart® SYBR® Green primers for rat cDNAs were provided by Sigma-Aldrich. The specificity of amplified genes was evaluated using the comparative cycle threshold method with the ABI Prism SDS software. The relative gene expression was calculated by using the $\Delta\Delta C_T$ analysis

for each sample, after normalization against β -actin gene expression. The air-exposed rat served as a reference and their mRNA expression was arbitrarily set as 1 unit.

2.6. Statistical analysis

For qPCR analysis, results are expressed as means \pm sem. Comparison between more than two groups were performed by one-way analysis of variance followed by Newman-Keuls multiple comparison post-hoc test. A student's *t* test was used to compare two groups. Differences were considered statistically significant when $p < 0.05$. Data analysis were performed with GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Biodiesel and diesel combustion emission analysis

The exhaust characterization was performed upstream (P1) and downstream (P2) of the DPF during the NEDC cycles. The results showed that DPF reduced the total PM concentrations for both fuels (Table S1). NO and NO_x concentrations were found higher in B30 than RFO exhausts, in contrast to CO concentrations, and the presence of DPF did not change the total gaseous hydrocarbon concentrations (Table S1). Concerning PAHs, particulate PAH concentrations measured upstream DPF (P1) ranged to 0.1-0.3 $\mu\text{g}/\text{m}^3$ for both fuels. PAH concentrations were next measured in the gas phase upstream and downstream of the DPF on XAD resin. The major PAHs identified were naphthalene > phenanthrene > fluoranthene and pyrene, whereas the measures of other PAHs were under the limit of detection (for example, benzo[a]pyrene < 1 ng/m^3). PAH concentration in B30 blend, ranging from 2.3-4.0 $\mu\text{g}/\text{m}^3$, was strongly decreased after DPF (P2) (Table 1), whereas such effect was less evident for the RFO fuel, likely due to a heterogeneity of PAH concentrations upstream DPF (P1) (Table 1). In fact, a reduced concentration was observed for PAHs, except for naphthalene which represents more than 80% of the measured PAHs. The heterogeneity of the PAH concentration upstream DPF may be due to a lower concentration of naphthalene during the first campaign measure, which remains to explain. However, it appears that naphthalene concentration was less important in exhaust from B30 fuel than from RFO fuel, whereas fluoranthene and pyrene concentrations seemed higher in B30 than in RFO fuels (data not shown). Alkane (C₈-C₄₀) concentrations upstream DPF (P1) were heterogeneous for both

fuels, but they clearly decreased after DPF (P2) for B30 but not for RFO fuels (Table 1). The measure of some volatile organic compound (VOC) (BTEX for benzene, toluene, ethylbenzene and xylene) showed a higher concentration of these compounds in exhaust from B30 blend than RFO fuel, as previously described (Beer et al., 2007), but the DPF has been clearly efficient to reduce their amount for both fuels (Table 1). The heterogeneity in BTEX concentrations measured in B30 emissions upstream the DPF was due to a xylene concentration highest in the second measure than in the first. Concerning aldehydes, no clear difference between RFO and B30 fuel exhausts was observed before DPF (P1). However, aldehyde concentrations (mostly formaldehyde and acetaldehyde) were highly increased after DPF (P2) for RFO, but were decreased for B30 blend (Table 1); this supports a strong impact of DPF on aldehyde release in the gas phase (Traviss et al., 2010). Altogether, our data show an efficiency of DPF to reduce all compounds analyzed in B30 fuel exhaust, whereas DPF did not permit to reduce the alkane and aldehyde concentrations in exhaust from RFO fuel. Furthermore, we showed that, after DPF, exhaust from RFO fuel contains more PAHs than that from B30 fuel, whereas B30 blend-exhaust contains more VOC such as BTEX.

3.2. Analysis of tissue and of biological parameters

Overall, there were no weight change between groups, except in the group of rats exposed to RFO before DPF (P1), which showed a significant reduction in weight-gain when compared to their respective filtered air controls (Table S2). Numeration of blood cells did not reveal any difference in rats between the different groups (data not shown). Histopathological examinations did not show major structural lung tissue alteration. It however revealed the presence of pigmented macrophages in the alveolar space of rats exposed to exhausts in P1, but not in the group of rats exposed to filtered air nor in the group of rats exposed to both fuels downstream the DPF (Figure S2). The histological scores of lung inflammation showed no or a mild inflammation in the lung of rats exposed to filtered air; they were higher in B30-exposed rats than those in rats exposed to RFO both downstream the DPF (100% of mild-moderate inflammation versus 44%) and upstream the DPF (83% of mild-moderate inflammation versus 67%), respectively (Figure S2).

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3.3. Genes differentially expressed in the lung of rats repeatedly exposed to RFO or B30 fuels

The lung gene response of rats exposed to RF0- and B30-exhaust upstream (P1) and downstream (P2) DPF were first compared, considering a pValue Log-ratio < 0.01 (Fig. 1). We did not applied a cut-off on the gene expression fold change, because the variations of gene expression levels were relatively low (not exceeding 2 fold). We first observed that the number of genes regulated by RF0 (137 genes) and B30 (153 genes) exhaust before DPF (P1) is quite similar (Fig. 1). The number of regulated genes downstream DPF (P2) was found to be higher than that before DPF (P1) for both fuels (Fig. 1). In addition, the number of genes regulated by exposure to B30 fuel after DPF (P2) (781 genes) is 1.8 fold higher than that regulated by exposure to RF0 fuel (426 genes) downstream DPF (P2) (Fig. 1). The comparison analyses are presented on the flow chart of the Fig. 1. Venn diagram reveals that only 6 genes are similarly regulated in rats exposed to RF0 and B30 fuel exhaust before DPF (P1), when compared to respective control rats, corresponding to about 4 % of the total number of RF0- and B30-regulated genes before DPF (P1) (Fig. 2A). Similarly, Venn diagram shows that only 35 genes are regulated in both RF0- and B30-exposed rats downstream DPF, corresponding to 8.2 % and 4.5 % of standard- and B30-regulated genes, respectively after DPF (P2) (Fig.2B). These shared regulated genes between RF0 and B30 in P1 and in P2 are listed in Table S3.

Because DPF has become mandatory on new diesel engines with the Euro V standard application since 2011 in European Union, we decided to use our data to gain insight into health effect of new depollution systems. For this purpose, we employed gene set enrichment analysis utilizing IPA on the RF0 (426 genes)- and the B30 (781 genes)-related regulated lung gene lists corresponding to a DPF downstream exposure (P2). The top 5 networks regulated by exposure to B30 blend exhaust downstream DPF are cancer, cellular assembly and organization, cellular movement, cell-to-cell interaction and tissue morphology (Table 2A), whereas the top 5 networks regulated by RF0 fuel exhaust downstream DPF are cellular development, cellular assembly and organization, lipid metabolism and hematological system development and function (Table 2B). In spite of the network “cellular assembly and organization” common to both fuel exposures, only two genes (ERK1/2 and Ppp2c) were found in both networks, showing a rather weak similarity of gene lists of this network.

We next evaluated and compared the top-ranked canonical pathways between RF0 and B30 fuels downstream the DPF (P2). The significance of all top five canonical pathways in B30-P2 (Fig. 3A) and in RF0-P2 (Fig. 3B) exceeds the threshold level for all of them. Our data show that exhaust exposure resulted in activation of some different specific signaling pathways. The

first three ranking canonical pathways regulated by B30 blend exhaust are “oxidative phosphorylation” (p-value = $1.02 \cdot 10^{-17}$ and ratio 0.25), “mitochondrial dysfunction” (p-value = $7.04 \cdot 10^{-15}$ and ratio 0.18) and “DNA methylation and transcriptional repression signaling” (p-value = $3.83 \cdot 10^{-4}$ and ratio 0.25) (Fig. 3A). The B30 blend-regulated genes associated to these first two pathways are all identical, plus three new genes in the “mitochondrial dysfunction” pathway such as GLRX2 (glutaredoxin 2, -1.18 fold), GPX7 (glutathione peroxidase 7, -1.34 fold) and APP (amyloid beta (A4) precursor protein, 1.21 fold) (Table S4). However, no activity pattern has been identified by z-score, which provides predictions about up or down processes, for these pathways. Concerning RF0 exposure, p-values of the identified canonical pathways are weaker than those of B30 exposure. So, the first three ranking canonical pathways concern “tRNA charging” (p-value = $5.81 \cdot 10^{-4}$ and ratio 0.12), “regulation of actin-based motility by Rho” (p-value = $1.13 \cdot 10^{-3}$ and ratio 0.07) and “Germ cell-Sertoli cell junction signaling” (p-value = $2.17 \cdot 10^{-3}$ and ratio 0.05) (Fig. 3B). Analysis of canonical pathways of B30 (P2) sorted by z-score also indicated a positive regulation of “regulation of actin-based motility by Rho” and “RhoA signaling”; thus RHOA gene, a ras homolog family member A, was found in common of the second to fifth-ranked pathways (Fig. 3B). Moreover, RAC2 (RAB8B, member RAS oncogene family) and PAK3 (p21 protein (Cdc42/Rac)-activated kinase 3) were two genes found in common in the second to fourth pathways.

Thus, there is no common top canonical pathway between RF0- and B30-exposed rats downstream DPF (P2). However, the analysis of the canonical pathways sorted by the z-score permits to identify 6 pathways in common (Fig. 4). Among them, “Integrin”, “IGF-1” and “Rac signaling” pathways seem the most activated in lungs of rats exposed to both fuels. Thus, 12 and 8 genes are associated with the “integrin signaling” pathway for B30 and RF0 fuels-exposed rats respectively, but without shared gene. In contrast, for the “IGF1 signaling” pathway, 2 genes (among the 7 and 4 genes regulated for the pathway in B30- and RF0-exposed rats, respectively), *i.e.*, the Protein Kinase CAMP-Dependent Type II Regulatory Subunit Alpha (PRKAR2A) and the Ribosomal Protein S6 kinase B1 (RPS6KB1), are in common. This latest gene RPS6KB1, regulated by the mTOR pathway, is also found in the “Rac signaling” pathway. However, the validation of the fold-expression of these genes by RT-qPCR analysis, confirms their weak regulation (between 0.8 to 1.3 fold) after an exposure of lungs to fuels (Table S5). Altogether, our data show a different gene response to RF0 and B30 in the lung of rats repeatedly exposed to these exhausts downstream DPF; however, it seems that pathways

involving membrane receptor (integrin and IGF-1) and Rac signaling, activated by both RF0 and B30 exhausts, could reflect the gaseous phase effects of these both exhausts.

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3.4. Analysis of the impact of DFP on gene expression in the lung of rats repeatedly exposed to B30 fuels

Since there is less data in the literature on biodiesel than diesel and since our p-value data for regulated genes in response to B30 are strongest than those obtained with exposure to RF0, we decided to focus on results obtained on lungs from rats exposed to B30 blend. The number of genes regulated by B30 in P2 (downstream DPF) was 5.1 fold higher than in P1 (upstream DPF) (781 versus 153 genes) (Fig. 5). Venn diagram also reveals that only 31 genes are common to P1 and P2 when compared to their respective air control, corresponding to about 20 % and 4% of P1- and P2-regulated genes by B30 exhaust (Fig. 5). Fig. 6 shows the first ten pathways, sorted by the p-value, in P1 (Fig. 6A) and in P2 (Fig. 6B) conditions for B30 exhaust exposure. Globally, the p-values and the percentages of genes involved in the canonical pathways from P1 are lower than those found in P2 (Fig. 6). The top five canonical pathways in the lungs of rats exposed to B30 upstream DPF (P1) are “acute phase response signaling”, growth hormone signaling”, IL-4 signaling”, “role of JAK2 in hormone-like cytokine signaling” and “IGF-1 signaling”; however, the percentage of affected genes to the total number of genes in a pathway is under 10%. Concerning the canonical pathways in the lungs of rats exposed to B30 exhaust downstream DPF (P2), the highest p-value concerns “oxidative phosphorylation” and “mitochondrial dysfunction” pathways, with a down-regulation of almost all genes presents in these pathways. These results were confirmed by a gene-enrichment and functional annotation analysis, using the DAVID software (data not shown). The p-values of the 8 other regulated pathways are almost all the same (Fig. 6B). In contrast to the two first pathways, genes involved in the “DNA methylation and transcriptional repression signaling”, ranking third, seems overexpressed. In contrast to what we observed for genes regulated by DPF upstream exposure to B30 exhaust (P1), the percentage of affected genes to the total number of genes in a pathway is equal or over 10% for the top five pathways regulated downstream DPF (P2). The “p70S6K signaling” is found present both in P1- and in P2- condition exposures (Fig. 6) with only one gene in common, i.e., RPS6KB1, previously identified also in RF0 exhaust exposure (P2).

Results of RT-qPCR analysis confirmed some microarray data. Thus, six selected genes from the two first regulated pathways exhibited a significant repression in the lungs of B30 (P2) exhaust-exposed rats (Fig.7). The mRNA expression of four genes of the mitochondrial NADH dehydrogenase [ubiquinone] 1 from the respiratory chain complex I (Ndufa2, Ndufa6, Ndubf2 and Ndufc1) and one of the complex V (ATP Synthase Subunit O (Atp5o)) were notably significantly reduced in comparison to the air control group. The down-expression of these genes was significant after exposure to B30 (P2) when compared both to control or to B30 (P1), except for Ndufa6 (Fig. 7). Even if the genes of the mitochondria respiratory chain have not been identified as differentially regulated in the lung samples of rat exposed to RF0 exhaust by the microarray analysis, RT-qPCR assays indicated that their mRNA expressions were also reduced in P2, in a similar way than those exposed to B30 exhaust in P2. In addition, the mRNA expression of glutathione peroxidase 7 (Gpx7), identified in the “mitochondrial dysfunction” pathway and whose biological role is to protect the organism from oxidative damage, was also significantly reduced in P2 groups exposed to B30 or RF0 exhausts (Fig. 7).

In order to better characterize the gene signature of the gaseous phase of B30 exhausts, we compared the top forty canonical pathways sorted first on the p-value and then on the z-score of B30 in P2. The results presented in the Table S6 show that the pathways with the highest score are “integrin-”, “oncostatin M-”, “VEGF-“, “EGF-“, “Rac-“ and “IGF-1-signaling” and the “remodeling of epithelial adherens junctions”. Five canonical pathways have a positive z-score ≥ 2 , suggesting an activation of these pathways by the gaseous phase of B30 blend exhaust. By contrast, the “mTOR signaling” pathway seems decreased. Because “integrin signaling”, “Rac signaling” and “IGF-1 signaling” ways have been previously identified in the lungs of rats exposed to RF0 exhaust downstream DPF (P2) such as to those exposed to B30 in P2 (Fig. 4), the others pathways cited could be specific to the gaseous phase of B30 exhausts.

4. Discussion

The present study is part of a research project aiming to better characterize the toxicity of biodiesel exhausts from modern engines, notably by comparing their effects to those of RF0 and the impact of the depollution system DPF. For this purpose, the whole genome RNA expression microarray covering 20 K of gene transcripts was used. The major results of the present study are as follow: 1) the levels of transcript deregulation in the lung of rats repeatedly exposed to diesel blend exhaust were rather weak (gene expression variations are

less than 2-fold), 2) the transcriptomic profile of changes due to B30 exhaust was different to that related to RFO exhaust, 3) a higher number of transcripts was deregulated by B30 exhaust downstream the depollution system than before DPF; 4) the lung transcript profile of B30-exposed rats was modified by the presence of the DPF, demonstrating an impact of the depollution system on lung tissue and 5) the repeated exposure to the gaseous phase of B30 seems associated to mitochondrial dysfunction.

At the animal level, repeated subacute exposure to RFO and B30 exhausts was not accompanied by apparent toxicity, except a lower weight gain for rats exposed to RFO upstream DPF. The absence of lung tissue injuries in rats exposed to diesel exhausts are in accordance with previous reports showing modest lung adverse effects of chronic soybean (Bass et al., 2015; Finch et al., 2002) or rapeseed oil-derived fuel exposure in rats (Magnusson et al., 2017). While no inflammatory pathway has been identified in the transcriptomic data from lung tissue, histological analysis revealed a mild to moderate inflammation in airways of rats exposed to diesel emissions, especially to B30 exhausts. As mitochondrial dysfunction may be linked to inflammatory responses (Lopez-Armada et al., 2013), our transcriptomic data support the histological observations. Because of such mild lung inflammation, we cannot argue that the genomic signatures reflect only the molecular changes within lung resident cell population, but also probably those of infiltrated cells.

The efficiency of the DPF was confirmed by the near absence of PM downstream the DPF and, at the histological level, by the absence of pigmented alveolar macrophages in lung of rats exposed downstream to this depollution system. Thus, BTEX concentrations were strongly reduced in exhausts downstream of DPF both for RFO and B30 fuels; a similar effect of DPF on B30 exhausts was also observed with alkanes and PAHs, whose concentrations decrease, as previously described for PAHs in B20 exhaust (Magnusson et al., 2017). By contrast, no DPF-related reduction of standard diesel pollutant emissions has been observed for alkanes and PAHs and, on the contrary, aldehyde concentrations were found strongly increased. This last result indicates that DPF may have changed the composition of exhausts. As formaldehyde may be an intermediate product from other aldehydes or hydrocarbons during oxidation reaction in catalyst, its decrease in exhausts from B30 in comparison to those of RFO may be related to the 30% dilution of diesel and thus to the lowest concentration of such initial compounds in B30 blend. Therefore, the depollution system DPF used in our experimental conditions mainly modifies the composition of B30 exhausts. However, despite such decreases of exhaust

concentrations, the number of regulated genes by B30 emissions was higher downstream than upstream the DPF. Such data were rather surprising since an opposite situation was previously observed in cardiac tissue of rats exposed to diesel emissions (Karoui et al, 2019), suggesting some specific tissue response to gas phase of exhaust or to remaining ultrafine particles.

Because of these modifications of exhaust composition after the DPF, a differential gene expression profile related to the presence of this depollution system was expected. Indeed, our transcriptomic data argue in favor of a specific response of rat lungs to B30 exhausts in function of the presence or not of DPF. Major canonical pathways identified are different, except the “p70S6K signaling”. However, the few number of genes associated to this pathway and the weak level of expression regulation do not permit to consider it as a global B30-related exhaust exposure signature. By contrast, “oxidative phosphorylation” and “mitochondrial dysfunction” pathways are only identified downstream of DPF; the down-regulation of genes associated to the mitochondrial respiratory chain, validated by RT-qPCR, confirms a gene-specific signature for lungs of rats exposed to exhaust emitted downstream the DPF. Indeed, the same profile of mitochondrial gene expression was found in lungs of rat exposed to RF0 downstream of DPF, suggesting that the gaseous fraction of exhaust may be the primary driver of the observed effects. The impact of the down-expression of genes from mitochondria respiratory chain should be evaluated by the measure of ATP production or by the oxygen consumption, to fully confirm the toxic effects of diesel exhausts towards mitochondria from lungs. Interestingly, previous studies demonstrated that adsorbed chemicals on DEP, such as PAHs, altered mitochondria functions (Xia et al., 2004) and that DEP extracts induce apoptosis of macrophages, via toxic effects on mitochondria, in association with reactive oxygen species (ROS) production (Hiura et al., 2000). In our experimental conditions, we did not however find any ROS production in lung tissues of rats exposed sub-chronically to RF0 or B30 (Douki et al., 2018). Nevertheless, a functional alteration of mitochondria respiratory chain was demonstrated in the myocardium of rats exposed to RF0 in the same conditions that those of the present study (Karoui et al., 2019), suggesting that a similar effect could also be expected in the lung. Also, the identification of the “oxidative phosphorylation” pathway in mice exposed to diesel exhaust (Stevens et al., 2008) and the role of mitochondria previously reported in pulmonary diseases such as asthma (Cloonan and Choi, 2016), whose exacerbations are often related to exposure to diesel exhaust (Alexis and Carlsten, 2014), support our data on lung mitochondrial dysfunction after a diesel exhaust exposure. In any

case, it is difficult to attribute the toxic effects on mitochondria of diesel exhausts to a specific pollutant that we have measured, at least in part because B30 but also RFO exhausts have altered mitochondria respiratory chain gene expression, whereas concentrations of BTEX, aldehydes, alkanes, PAHs, NO, NOX and CO have been found to differ downstream of DPF.

By contrast, the differences in concentrations of gaseous pollutants may explain the poor overlapping of transcript expression that we observed between both fuels. So, the addition of 30% of rapeseed methylester was efficient to reduce aldehyde and alkane concentrations (Bakeas and Karavalakis, 2013) as well as those of PAHs, but not BTEX concentrations. The nature of PAHs was found to differ between RFO and B30 exhausts, i.e., naphthalene concentration was higher in RFO than B30, whereas the fluoranthene and pyrene concentrations were higher in B30 than RFO. So, the greater number of regulated genes in lung of rat exposed to B30 compared to those exposed to RFO may be related at least in part to the higher concentrations of fluoranthene, pyrene, NO, NOx and VOCs such as BTEX, especially of xylene. In spite of the low numbers of genes regulated in common in response to RFO and B30 exhaust exposure downstream DPF (P2), that can be explain by the difference in PAH nature and the lowest concentration of BTEX (in RFO versus B30), three pathways (integrin-, IGF-1- and Rac-signaling) were considered as activated for both fuel exhausts, which could represent the gaseous phase profile for both diesel exhausts. Integrins are major membrane proteins involved in cell-cell or cell-matrix interactions and integrin engagement regulates the activity of several members of small GTPases such as RhoA or Rac1, which control the cell contraction and migration. Integrin expression was altered by an exposure to diesel exhausts in human airway epithelial cells (Doornaert et al., 2003; Le Vee et al., 2016) and also in lung biopsies of human volunteers after a short term exposure to diesel exhaust (Salvi et al., 1999), suggesting that fuel exhausts may alter lung tissue homeostasis through impairing integrin-mediated cell adhesion. Insulin like Growth Factor (IGF-)-1 signaling pathway regulates multiple cellular processes, including proliferation, differentiation, energy metabolism or glucose homeostasis. IGF-1R is highly expressed in lung epithelial cells, alveolar macrophages and smooth muscle (López et al., 2016) and binding of ligands to IGF-1 receptor leads to its activation and the subsequent activation of Shc/RAS/RAF/ERK-MAPK or IRS/PI3K/AKT/mTOR pathways, which promotes cell proliferation. Such signaling pathway activation is consistent with (i) the increased level of IGF signaling genes were increased in the lung of mice exposed to naphthalene, a PAH known to alter airway epithelium and present in

diesel fuel exhaust (López et al., 2016), and (ii) the increase of IGF-1 plasma levels in traffic policemen exposed to urban pollutants (Tomei et al., 2004).

Our study has some limitations. First, our transcriptomic analysis was only done after a repeated exposure to diesel exhausts, without comparison to a short/acute exposure that would be informative on the kinetic of transcript expression profile of lung tissue exposed to diesel blend. Second, this transcriptomic study could have been carried with a more recent and more efficient approaches, such as the next-generation sequencing (NGS), but when this study began, NGS was quite expensive and not so spread. Third, it would have been interesting to complete our study on the inflammatory response after exposure to diesel emissions with an analysis of inflammatory secreted markers in the broncho-alveolar lavages; this was however not possible to do it, because the full lung of each animal was required for other studies performed by our research consortium (Douki et al., 2018).

5. Conclusion

In summary, using a genomic analysis, we have identified different genes and pathways regulated in lungs of rats repeated exposed to standard diesel or to 30% rapeseed methyl ester. In accordance with previous data published by our research consortium (Douki et al., 2018 ; Karoui et al, 2019), we confirmed that the gaseous phase may have a higher impact, but remaining anyway moderate, on lung tissue than the complete exhausts.

Conflicts of interests

The authors have no conflict to interest to declare.

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Author contributions

499 Conceived and designed the experiments: VL. CM. TD. FS. OF. Performed the experiments: VL.
500 CM. MJ. FC. PG. Contribute to reagents/materials/analysis tools: CC. DP. Wrote the article: VL.
501 OF.

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Legends of Figures:

Figure 1: Flow chart of exposure conditions and microarray analysis of rat lung tissue indicating the essential experimental and data analysis. For each exposure campaign, three groups of six rats were exposed to filtered air or to reference diesel (RF0) or 30% rapeseed biofuel (B30), upstream and downstream of diesel particle filter (DPF). After RNA extraction and validation of RNA quality, lung gene expression profiles were determined using GeneChip® RAGENE 2.0 ST Arrays.

Figure 2: Presentation by Venn Diagram of the differentially expressed genes in lungs of rats exposed to exhausts upstream DPF (P1) (A) and downstream DPF (P2) (B), when compared to their respective filtered air controls. Genes were selected on the basis of a p-Value Log-ratio ≤ 0.01

Figure 3: Five top canonical pathways regulated by B30 exhaust in P2 versus air control (A) or regulated by RF0 exhaust in P2 versus air control (B), sorted by the p-value. Bars represent -log(p-value) of affected genes in the selected pathways; yellow lines represent the ratio of affected genes to the total number of genes in a pathway. Threshold (yellow dotted lines) denote the p=0.01 level.

Figure 4: Comparison of the 6 top canonical pathways sorted by z-score between rat lungs exposed to RF0 and B30 fuel exhaust downstream DPF (P2).

Figure 5: Presentation by Venn diagram of the number of genes differentially expressed in lungs of rats exposed to B30 upstream DPF (in P1) and downstream DPF (in P2), when compared to the filtered air control. Genes were selected on the basis of a p-Value Log-ratio ≤ 0.01 .

Figure 6: Ten top canonical pathways of B30 exhaust upstream DPF (P1) (A) and downstream DPF (P2) (B). Bars represent the percentage of affected genes to the total number of genes in a pathway; in green, genes down-regulated and in red genes, up-regulated in the pathway. Yellow lines represent -log(p-value) of affected genes in the selected pathways.

673 **Figure 7:** RT-qPCR analysis of gene expression in lungs from rats exposed to RFO or B30
674 exhausts upstream (P1) or downstream DPF (P2). Analyzed genes were selected from the
675 “mitochondrial dysfunction” pathway. Data are expressed as -fold change gene expression
676 when compared to control and are the means \pm SEM of 6 lung samples. t-Test analysis: *:
677 $p < 0.05$; **: $p < 0.01$ vs their respective air control identified by a dashed line. #: $p < 0.05$; ##:
678 $p < 0.01$.

Table 1: BTEX (Benzene, Toluene, Ethylbenzene, Xylene), aldehydes, alkanes (from C8 to C40) and Polycyclic Aromatic Hydrocarbons (PAHs) concentrations in $\mu\text{g}/\text{m}^3$ in samples from RF0 or B30 exhausts collected upstream of the diesel particle filter (DPF) (P1) or downstream the DPF (P2). Two measures were done for each exposure campaign.

Chemicals ($\mu\text{g}/\text{m}^3$)	Standard fuel (RF0)					Fuel mix (B30)				
	P1 measure	P1 measure	P2 measure	P2 measure	Range of Ratio P2/P1	P1 measure	P1 measure	P2 measure	P2 measure	Range of Ratio P2/P1
BTEX	45,8	53,6	4,17	7,80	0,09-0,15	82,2	218,9	24,2	124,7	0,3-0,6
aldehydes	37,3	44,2	182,9	194,3	4,4-4,9	13,5	36,1	14,2	11,3	0,3-1,1
alkanes (C8-C40)	124,3	341,5	438,3	133,9	0,4-3,5	368,0	177,8	63,9	66,4	0,2-0,4
PAHs	1,02	9,89	5,79	3,51	0,4-5,7	4,04	2,38	0,58	0,07	0,03-0,14

Figure 1:

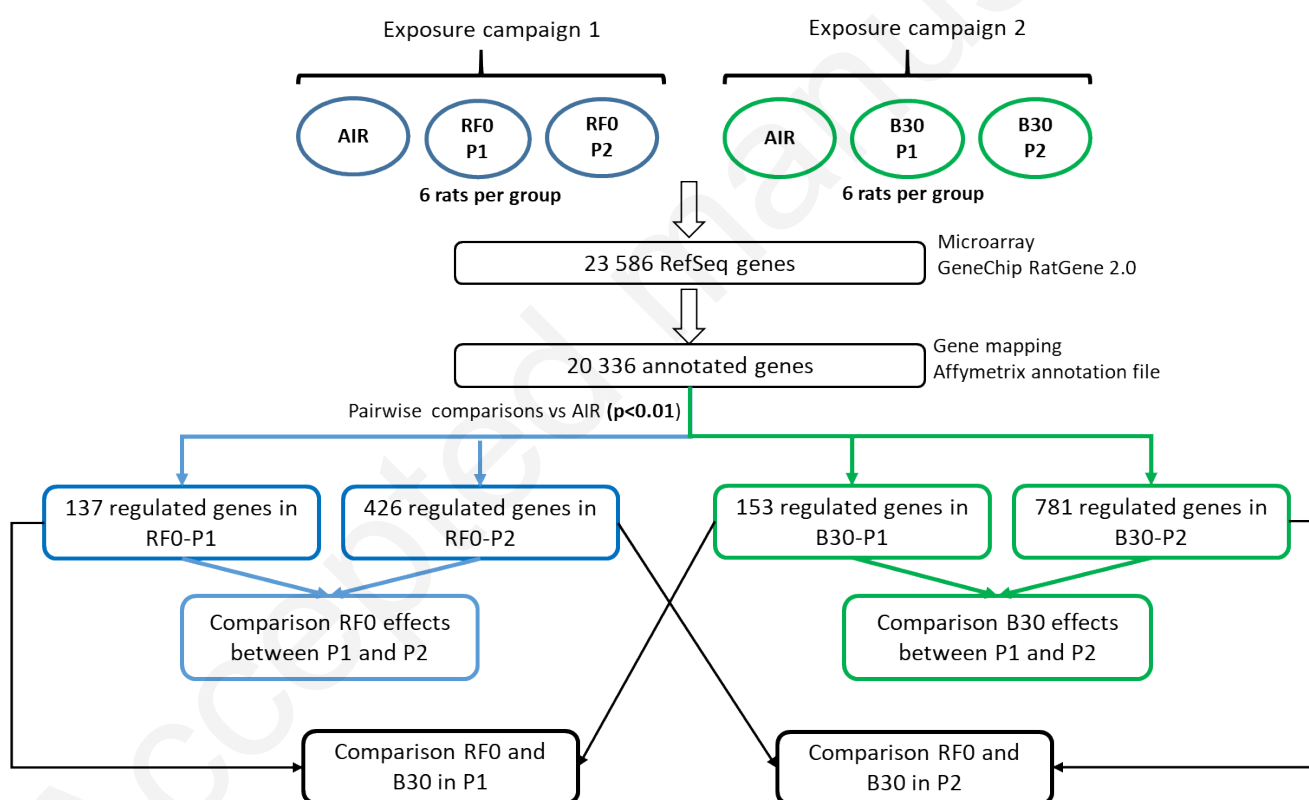


Figure 2 :

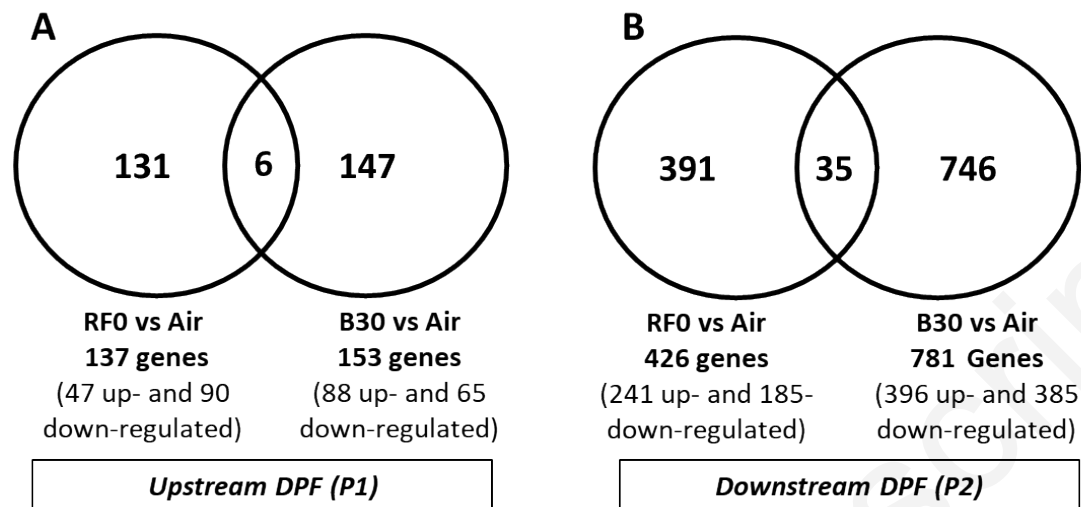
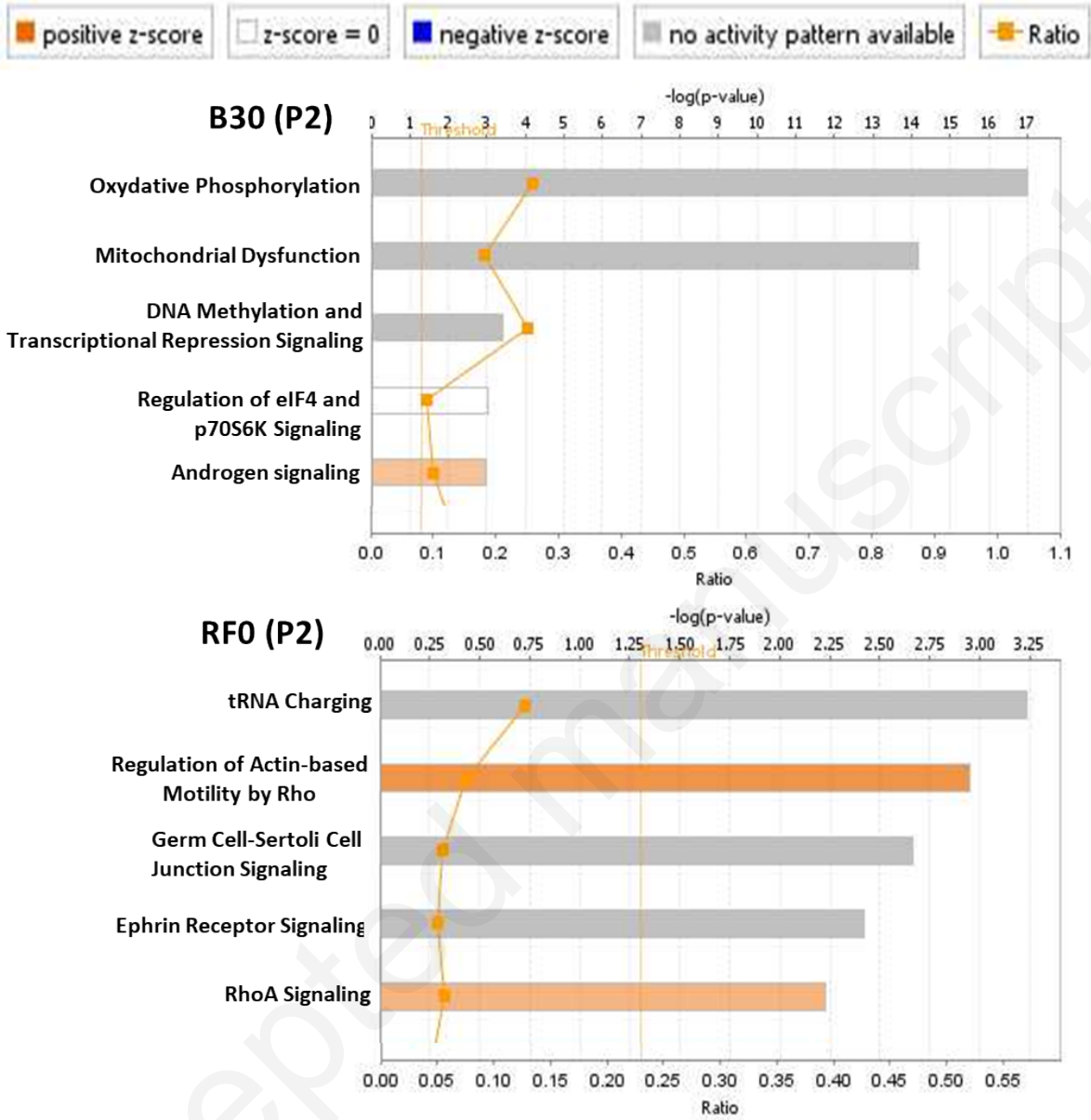


Table 2: Top Network of B30 P2 (A) and RF0 P2 (B)

A	Top Diseases and Functions (B30 P2 versus air control)		Score	Diff expressed genes in pathway
	1	Cancer, Gene Expression, Cellular Development	45	33
	2	Cellular Assembly and Organization, Cellular Function and Maintenance, Cell Death and Survival	34	28
	3	Cellular Movement, Cell-To-Cell Signaling and Interaction, Hair and Skin Development and Function	34	27
	4	Cell-To-Cell Signaling and Interaction, Tissue Development, Cellular Assembly and Organization	30	25
	5	Tissue Morphology, Metabolic Disease, Cellular Development	30	26
B	Top Diseases and Functions (RF0 P2 versus air control)		Score	Diff expressed genes in pathway
	1	Cellular Development, Nervous System Development and Function, Skeletal and Muscular System Development and Function	37	26
	2	Cellular Assembly and Organization, Cellular Development, Cellular Growth and Proliferation	33	24
	3	Cellular Assembly and Organization, Cellular Function and Maintenance, Protein Synthesis	31	23
	4	Lipid Metabolism, Small Molecule Biochemistry, Developmental Disorder	27	21
	5	Hematological System Development and Function, Inflammatory Response, Tissue Morphology	22	18

692 **Figure 3 :**



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Figure 4:

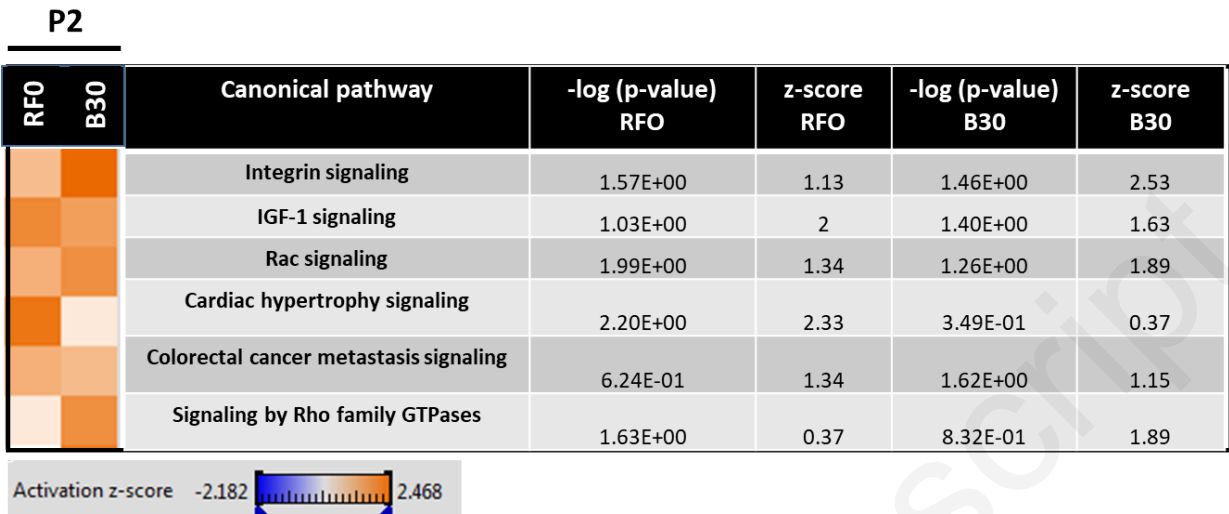
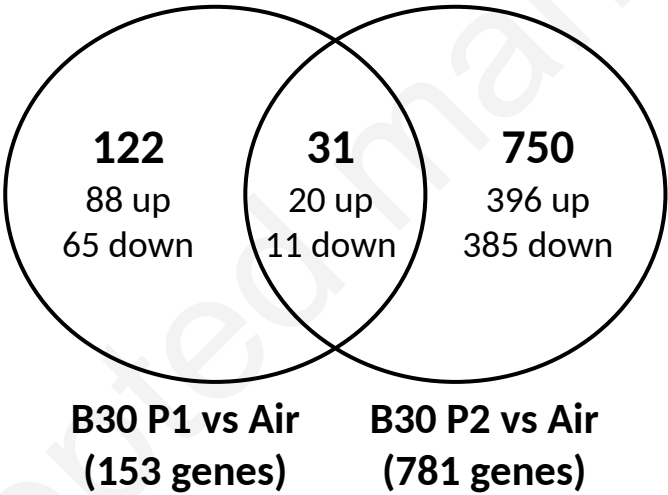
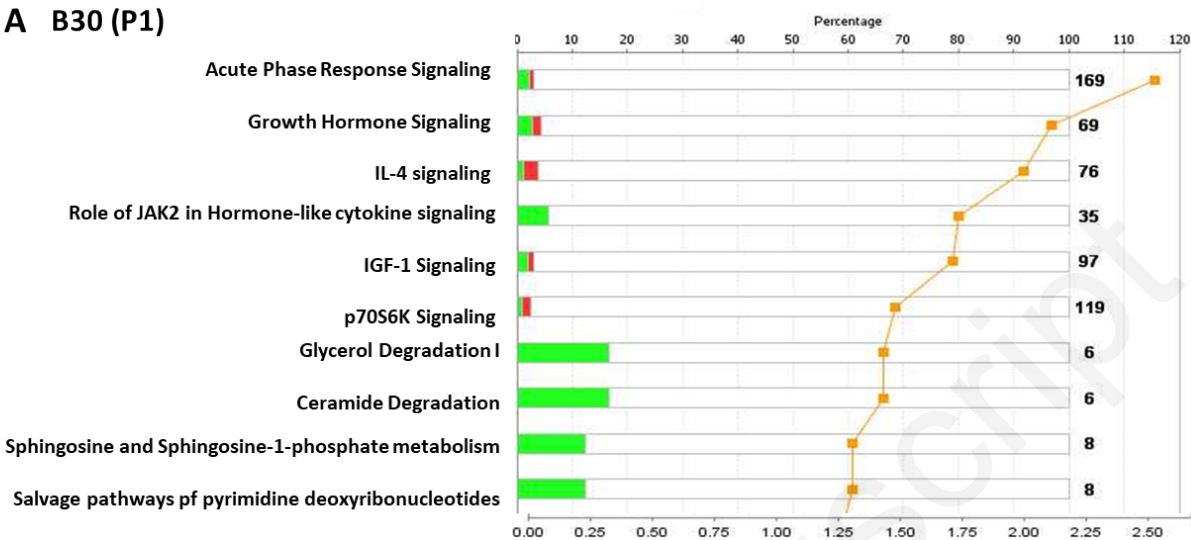


Figure 5:

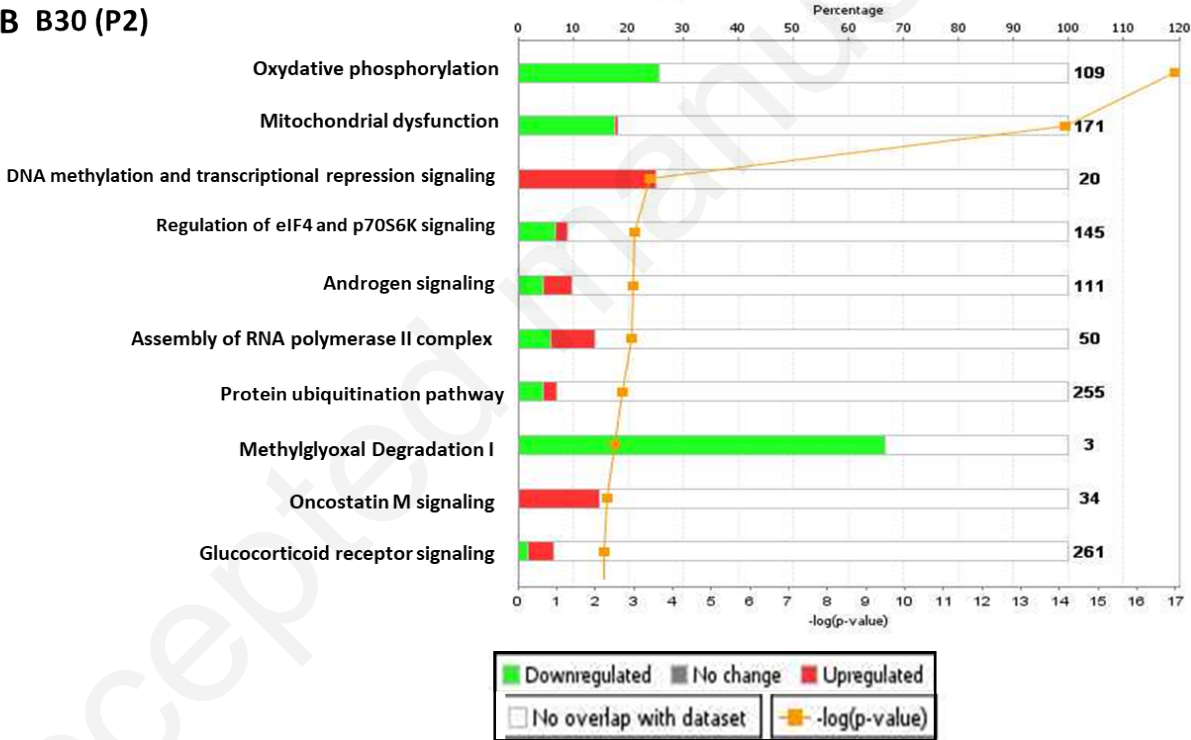


697 **Figure 6:**

A B30 (P1)

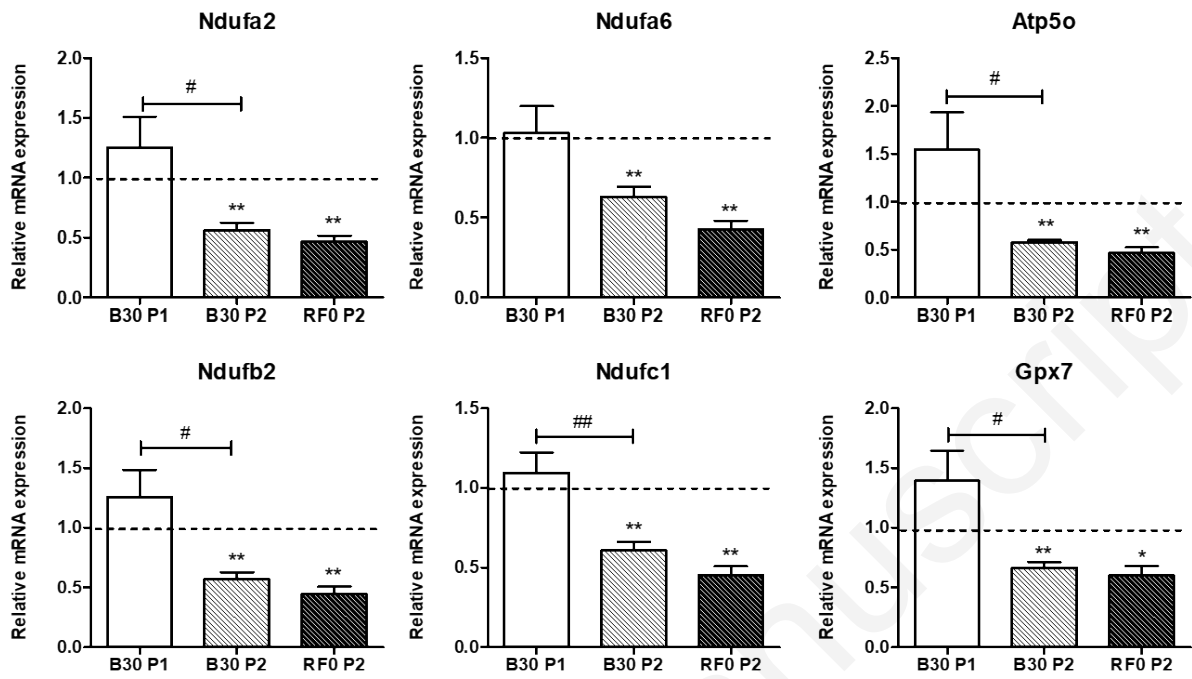


B B30 (P2)



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700 **Figure 7:**



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Table 1 : BTEX (Benzene, Toluene, Ethylbenzene, Xylene), aldehydes, alkanes (from C8 to C40) and Polycyclic Aromatic Hydrocarbons (PAHs) concentrations in $\mu\text{g}/\text{m}^3$ in samples from RF0 or B30 exhausts collected upstream of the diesel particle filter (DPF) (P1) or downstream the DPF (P2). Two measures were done for each exposure campaign.

Chemicals ($\mu\text{g}/\text{m}^3$)	Standard fuel (RF0)					Fuel mix (B30)				
	P1 measure	P1 measure	P2 measure	P2 measure	Range of Ratio P2/P1	P1 measure	P1 measure	P2 measure	P2 measure	Range of Ratio P2/P1
BTEX	45,8	53,6	4,17	7,80	0,09-0,15	82,2	218,9	24,2	124,7	0,3-0,6
aldehydes	37,3	44,2	182,9	194,3	4,4-4,9	13,5	36,1	14,2	11,3	0,3-1,1
alkanes (C8-C40)	124,3	341,5	438,3	133,9	0,4-3,5	368,0	177,8	63,9	66,4	0,2-0,4
PAHs	1,02	9,89	5,79	3,51	0,4-5,7	4,04	2,38	0,58	0,07	0,03-0,14

Fig.1

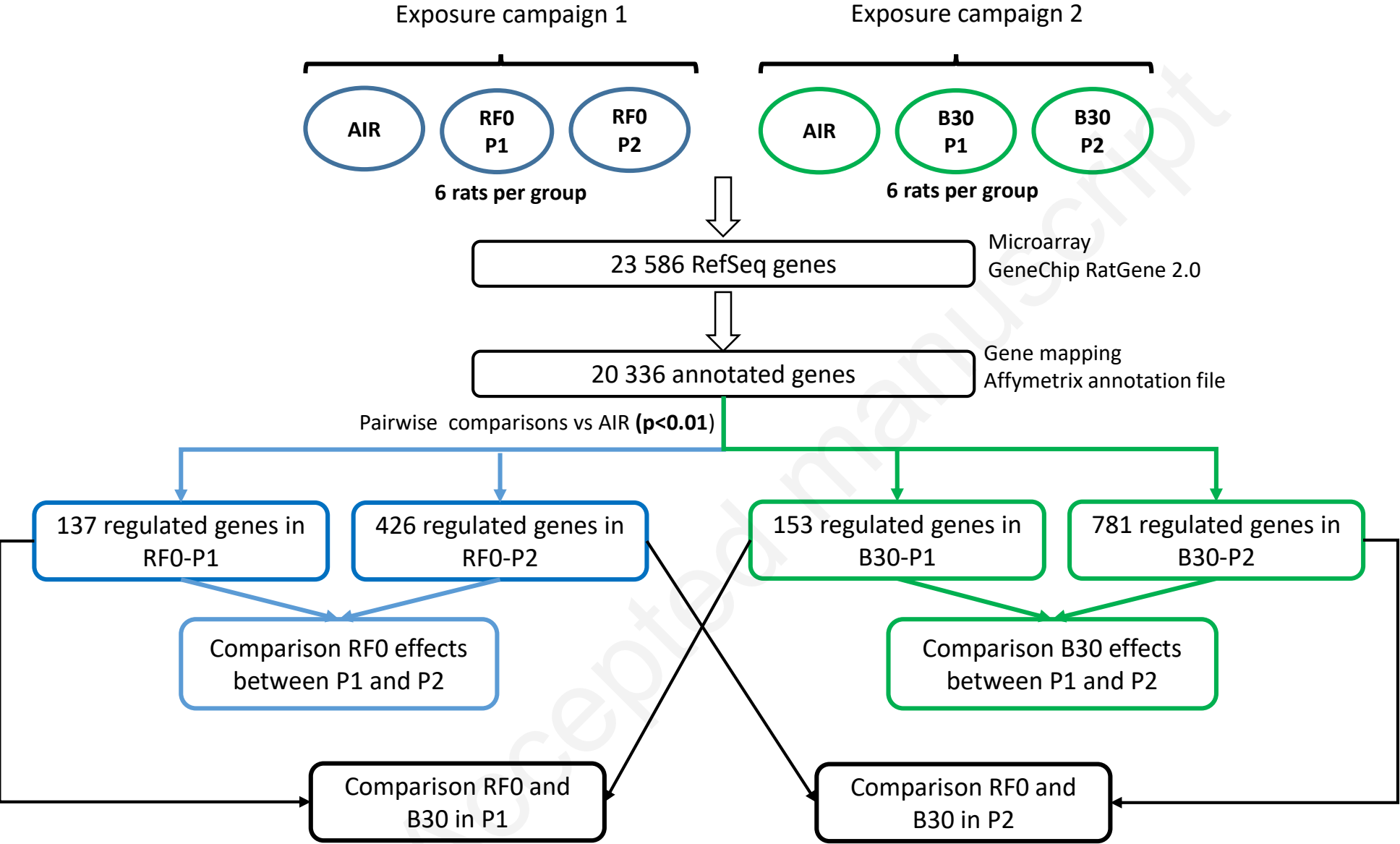


Fig.2

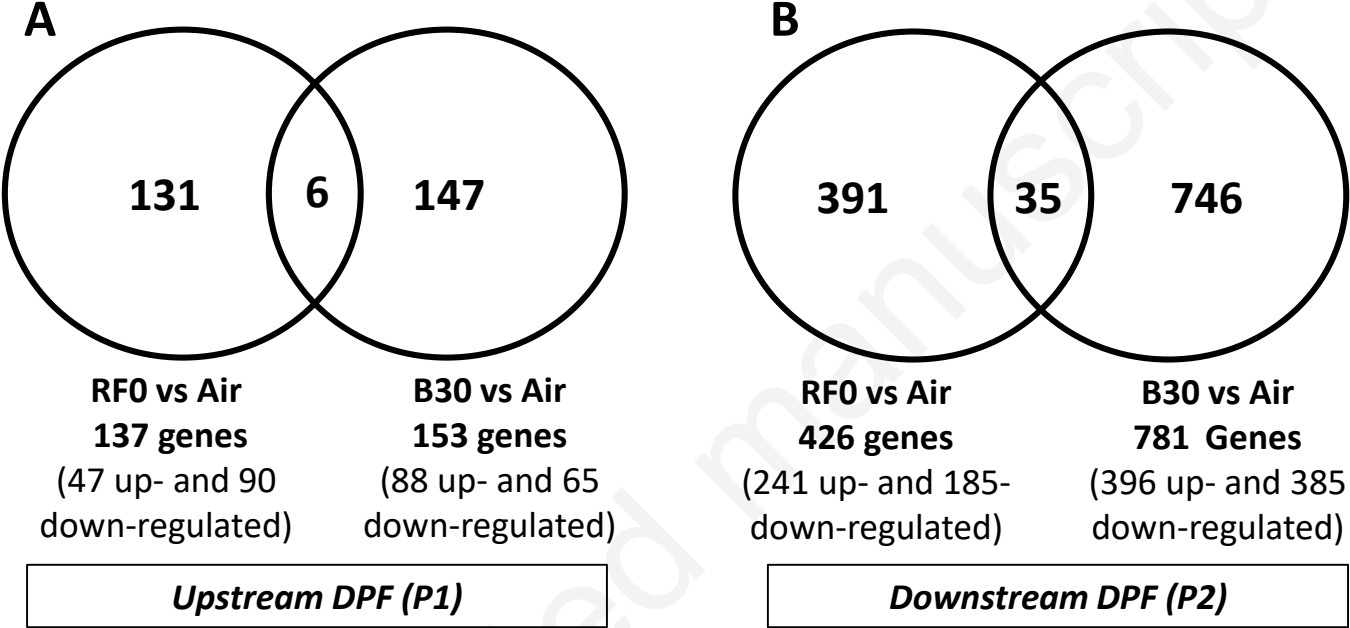


Table 2: Top Network of B30 P2 (A) and RF0 P2 (B)

A	Top Diseases and Functions (B30 P2 versus air control)	Score	Diff expressed genes in pathway
1	Cancer, Gene Expression, Cellular Development	45	33
2	Cellular Assembly and Organization, Cellular Function and Maintenance, Cell Death and Survival	34	28
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4	Cell-To-Cell Signaling and Interaction, Tissue Development, Cellular Assembly and Organization	30	25
5	Tissue Morphology, Metabolic Disease, Cellular Development	30	26
B	Top Diseases and Functions (RF0 P2 versus air control)	Score	Diff expressed genes in pathway
1	Cellular Development, Nervous System Development and Function, Skeletal and Muscular System Development and Function	37	26
2	Cellular Assembly and Organization, Cellular Development, Cellular Growth and Proliferation	33	24
3	Cellular Assembly and Organization, Cellular Function and Maintenance, Protein Synthesis	31	23
4	Lipid Metabolism, Small Molecule Biochemistry, Developmental Disorder	27	21
5	Hematological System Development and Function, Inflammatory Response, Tissue Morphology	22	18

Fig.3

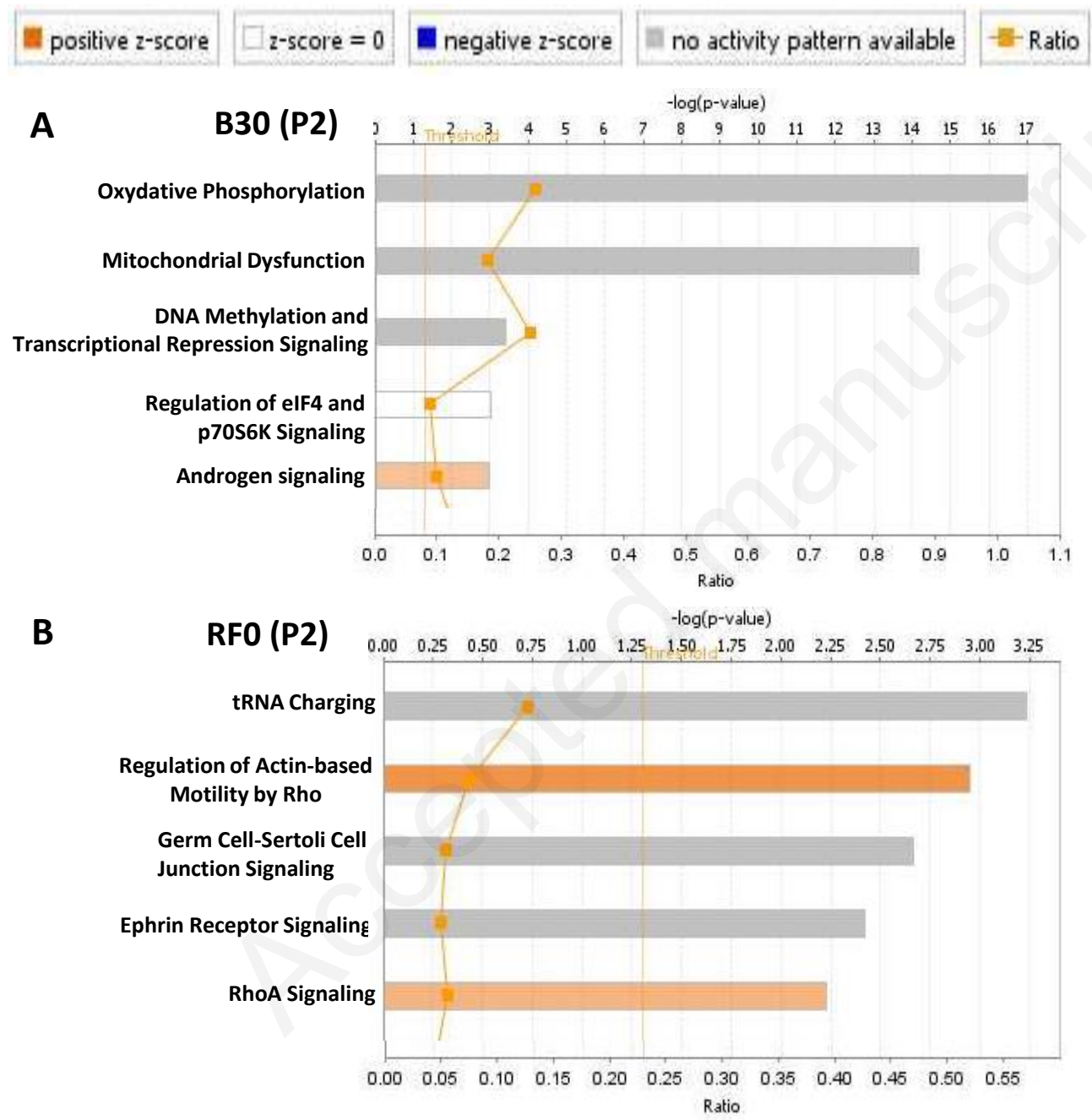


Fig.4

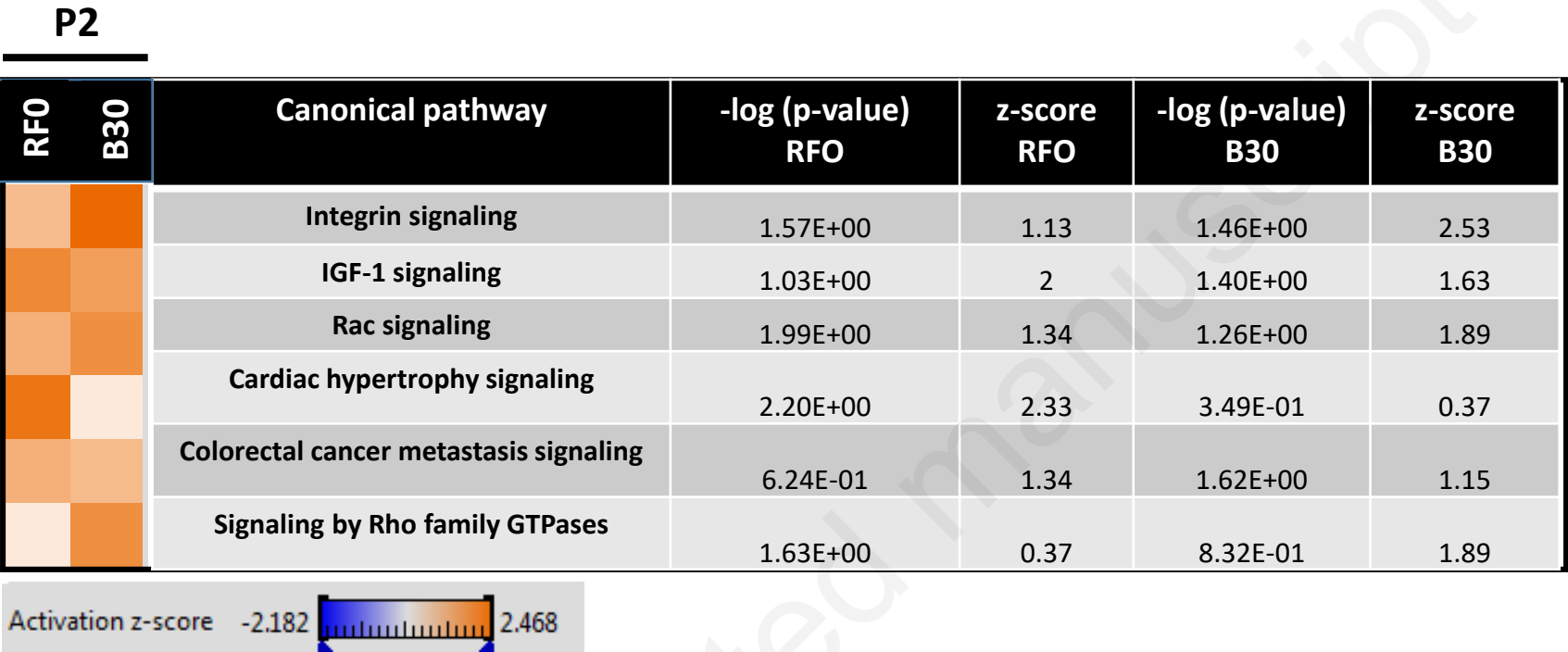


Fig.5

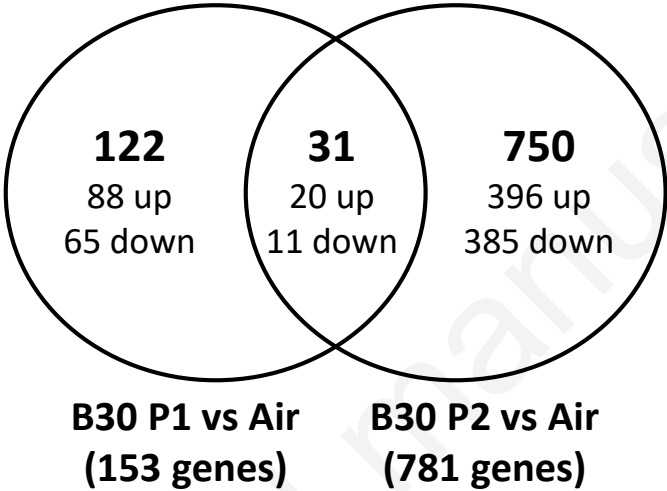
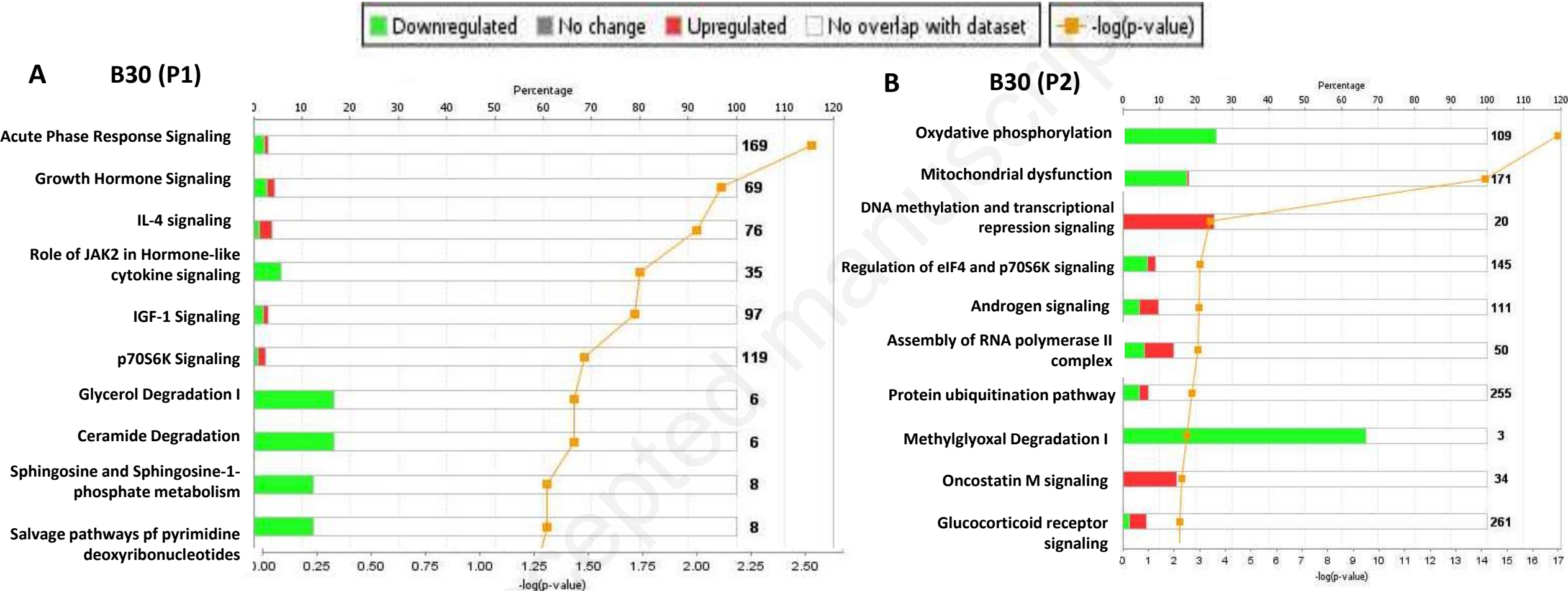
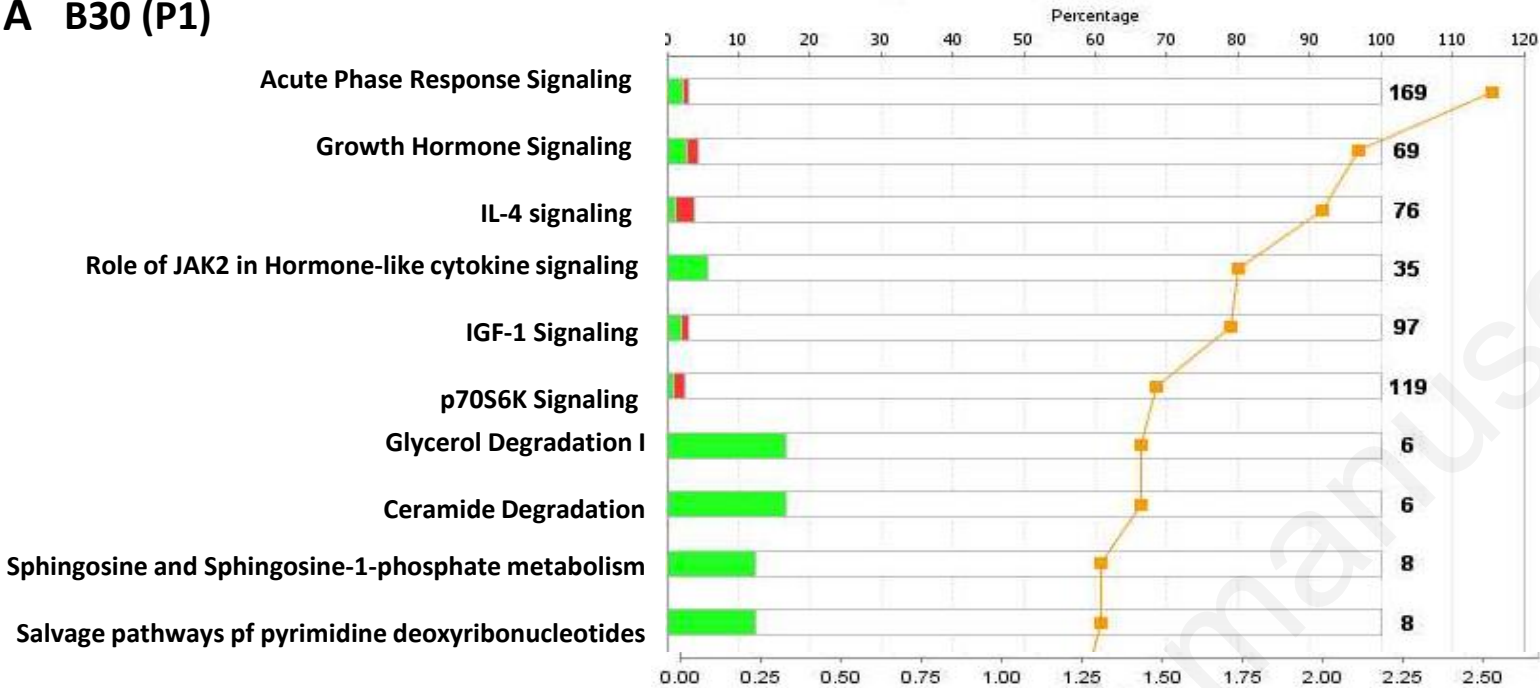


Fig.6



A B30 (P1)



B B30 (P2)

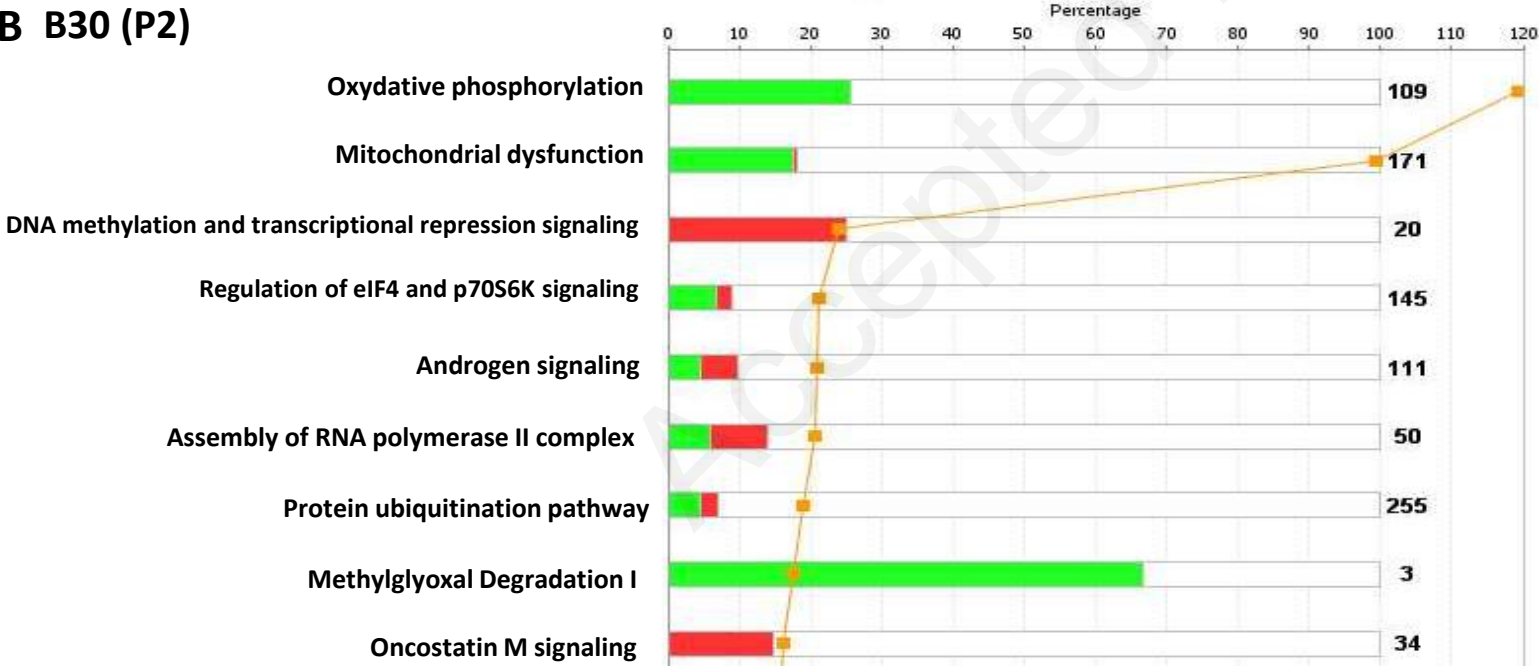
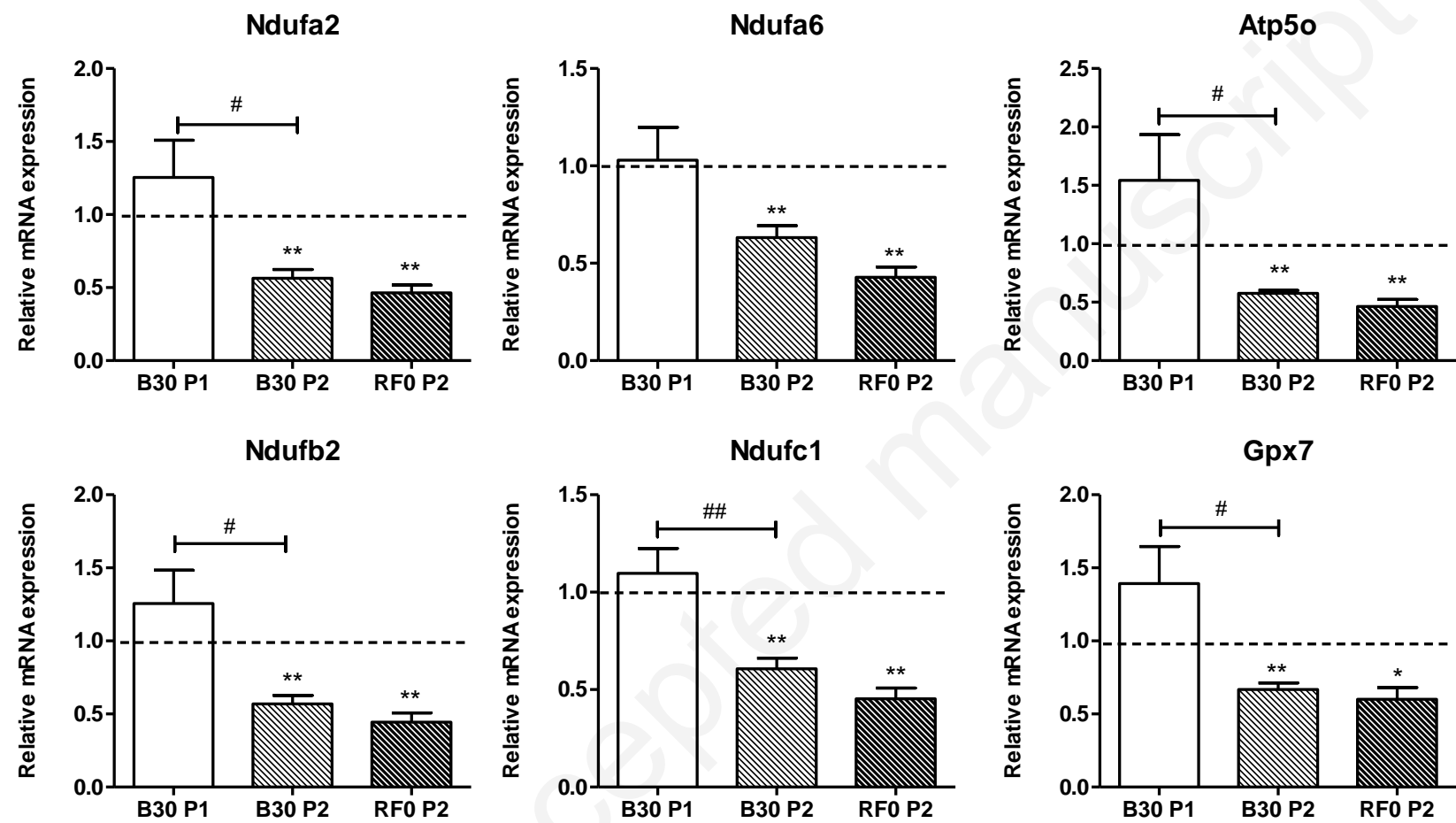


Fig.7



Declaration of interests

☐ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Valérie Lecureur : Conceptualization, Project administration, Data curation, Investigation, Formal analysis, writing. **Christelle Monteil** : Conceptualization, Project administration, Investigation, Formal analysis. **Marie Jaguin** : Methodology, Validation. **Fabrice Cazier** : Methodology, Investigation, Formal analysis. **David Preterre** : Conceptualization, Investigation. **Cécile Corbière** : Methodology, Investigation. **Pierre Gosset** : Methodology, Investigation, Formal analysis. **Thierry Douki** : Conceptualization, Project administration, Funding acquisition. **François Sichel** : Conceptualization, Project administration, Funding acquisition. **Olivier Fardel** : Project administration, Investigation, writing

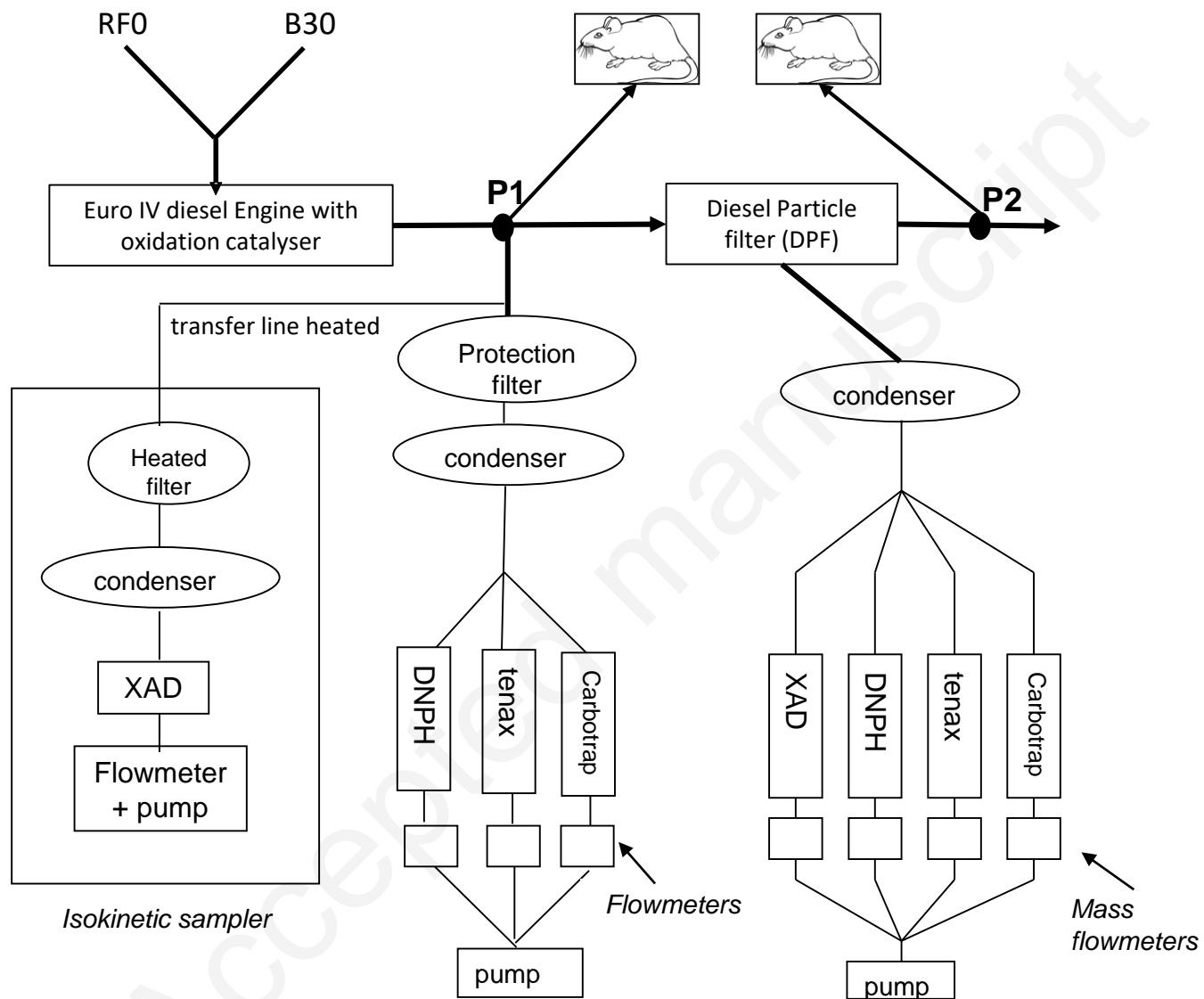


Figure S1: Set-up for the exposure of animals to the exhausts of a diesel engine collected either upstream (P1) or downstream (P2) of the diesel particle filter (DPF) and for collection and measurements of exhausts.

Table S1 : Gaseous pollutants concentrations and total PM mass concentration measured in the raw exhaust. Gaseous pollutants monitored continuously in the exhaust emitted by a diesel engine used under driving cycles NEDC. The values indicate the mean of concentration measured during 9 NEDC cycles during 3h of exposure.

Gaseous pollutants concentrations	RF0		B30		B30/RF0	B30/RF0
	P1	P2	P1	P2	P1	P2
NO, ppmV	17	15	34	35	2	2,3
NOX, ppmV	30	28	83	82	2,8	2,9
Total gaseous hydrocarbons, ppmV	26	26	21	15	0,8	0,6
CO, ppmV	103	95	31	28	0,3	0,3
PM concentrations (mg/m ³)	24	<0,1	23	<0,1	nd	nd

Table S2 : Body weight of rats before and after 3 weeks (3h/day and 5 days/week) of exposure to Air, RF0 or B30 exhausts, upstream (P1) or downstream (P2) to the particles filter (DPF). Data are the means \pm sem (n=6 per lots). The data in brackets represents the percentages of weight gain of the rats. *: p<0.05 vs their respective Air control .

	weight basal	weight after 3 weeks		
		Air	P1	P2
RF0	299 \pm 6	436 \pm 14 (+ 45 %)	396 \pm 17* (+ 31 %)	420 \pm 11 (+ 43 %)
B30	269 \pm 3	364 \pm 15 (+ 37 %)	337 \pm 12 (+28 %)	338 \pm 9 (+ 30 %)

Figure S2: Histological analysis of rat lung tissues. The table shows the histological score of lung inflammation and the aspect of alveolar macrophages (pigmented or not). Staining of lung (left upper lobe) (x400) by HES of rat exposed to filtered air (A) or to RF0-P1 (B) showing non pigmented macrophages (A) and pigmented macrophages (B) (black arrow).

		Histological score of lung inflammation				Presence of pigmented macrophages in bronchi
		1 = No	2 = Mild	3 = moderate	4 =severe	
campaign 1- RF0	Filtered Air (n=6)	50%	50%	0%	0%	0%
	RF0-P1 (n=6)	66%	22%	22%	0%	100%
	RF0-P2 (n=6)	33%	17%	50%	0%	0%
campaign 2- B30	Filtered Air (n=6)	66%	34%	0%	0%	0%
	B30-P1 (n=6)	0%	50%	50%	0%	100%
	B30-P2 (n=6)	17%	50%	33%	0%	0%

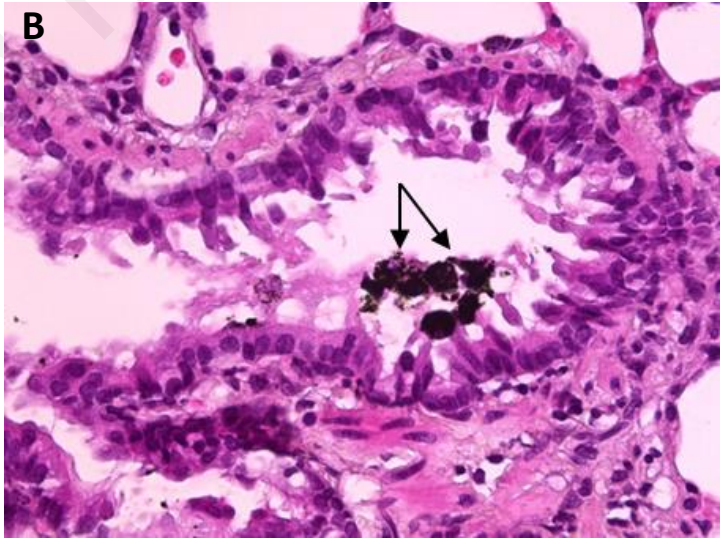
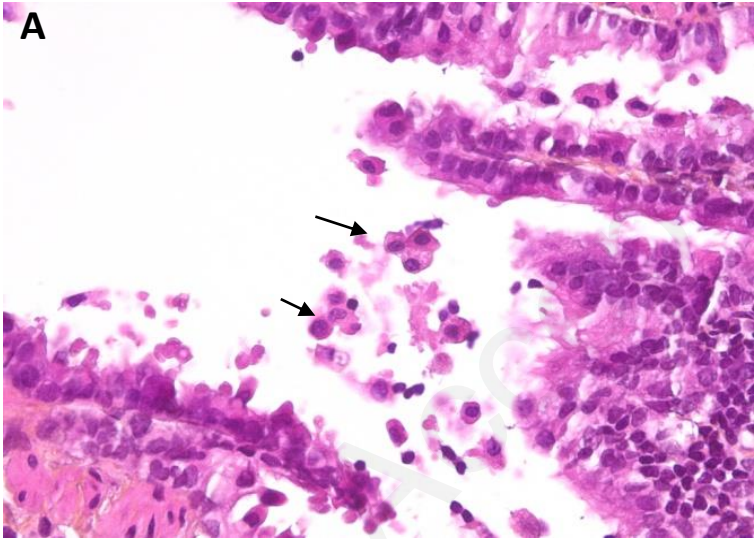


Table S5 : Comparison of mRNA expression variation (in fold) between transcriptomic array values and RT-qPCR data.

Fold expression	RF0 in P2		B30 in P2	
	Array	PCR	Array	PCR
RPS6KB1	1.1	0.8	1.11	0.85
PRKAR2A	1.33	0.87	1.21	1.05

Table S6 : List of the canonical pathways regulated by B30 exhaust exposure in P2/DPF downstream condition, with **the** z-score. This list was obtained by sorting the forty first canonical pathways (classed in function of their p-values) by their z-score. In bold, canonical pathways also identified in RFO (P2).

Canonical Pathways of B30 in P2 on the forty first pathway sorted first by the p-value and then by the z-score.				
Ingenuity Canonical Pathways	-log(p-value)	Ratio	z-score	Molecules
Integrin Signaling	1.46E+00	5.94E-02	2.53	ROCK1,ARHGAP5,SRC,ITGA3,ARF5,Ppp1r12a,RHOC,ITGA6,TLN1,ACTN4,PDGFB,ACTN1
Oncostatin M Signaling	2.33E+00	1.47E-01	2.23	TIMP3,JAK1,STAT3,PLAU,STAT5B
VEGF Signaling	1.53E+00	7.69E-02	2.23	ROCK1,EIF2S2,SRC,HIF1A,ACTN4,ACTN1,EIF1AX
EGF Signaling	1.44E+00	8.93E-02	2.23	RPS6KB1,SRC,JAK1,JUN,STAT3
Remodeling of Epithelial Adherens Junctions	1.62E+00	8.82E-02	2	SRC,CDH1,ACTN4,IQGAP1,ACTN1,CTNND1
Rac Signaling	1.26E+00	6.73E-02	1.89	RPS6KB1,ITGA3,JUN,CYFIP1,NCF2,PIP4K2B,IQGAP1
IGF-1 Signaling	1.40E+00	7.22E-02	1.63	RPS6KB1,JAK1,JUN,CTGF,PRKAR2A,STAT3,PRKAG1
PCP pathway	1.26E+00	7.94E-02	1.34	ROCK1,JUN,FZD4,WNT10A,FZD6
Leukocyte Extravasation Signaling	1.52E+00	6.06E-02	1.26	ROCK1,ARHGAP5,SRC,TIMP3,ITGA3,CYBA,NCF2,ITGA6,RASSF5,ACTN4,ACTN1,CTNND1
Colorectal Cancer Metastasis Signaling	1.62E+00	5.93E-02	1.15	SRC,JAK1,RHOC,GNB2L1,PRKAR2A,STAT3,PRKAG1,CDH1,JUN,FZD4,MSH2,WNT10A,FZD6,RALGDS
Androgen Signaling	2.98E+00	9.91E-02	1	POLR2G,SRC,CCNH,POLR2A,JUN,GNB2L1,PRKAR2A,GTF2E2,GTF2F1,GTF2A1,PRKAG1
mTOR Signaling	2.06E+00	6.95E-02	0.44	RPS6KB1,RPS4Y1,EIF3F,FAU,RPS16,RHOC,RPS27L,EIF3A,HIF1A,RPS5,EIF4E,PRKAG1,RPSA