Investigating the molecular processes behind the cell-specific toxicity response to titanium dioxide nanobelts

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Abstract

Background: Whereas several engineered nanomaterials are able to incite toxicological effects, the underlying molecular processes are understudied. And the varied physicochemical properties complicate toxicological predictions. Gene expression data allow us to study the cell-specific responses of individual genes, whereas their role in biological processes is harder to interpret. An overrepresentation analysis allows us to identify enriched biological processes and link the experimental data to these, but still prompt broad results which complicates the analysis of detailed toxicological processes. We demonstrated a targeted filtering approach to compare the cell-specific effects of two concentrations of the widely used nanomaterial titanium dioxide (TiO2)-nanobelts.

Methods: We compared public gene expression data generated by Tilton et al. from colon endothelium cells (Caco2), lung endothelium cells (SAE), and monocytic like cells (THP1) after 24-hour exposure to low (10 µg/ml) and high (100 µg/ml) concentrations of TiO2-nanobelts. We used pathway enrichment analysis of the WikiPathways collection to identify cell and concentration-specific affected pathways. Gene sets from selected Gene Ontology terms (apoptosis, inflammation, DNA damage response and oxidative stress) highlighted pathways with a clear toxicity focus. Finally, pathway-gene networks were created to show the genetic overlap between the altered toxicity-related pathways.

Results: All cell lines showed more differentially expressed genes after exposure to higher concentration, but our analysis found clear differences in affected molecular processes between the cell lines. Approximately half of the affected pathways are categorized with one of the selected toxicity-related processes. Caco2 cells show resilience to low and high concentrations. SAE cells display some cytotoxic response to the high concentration, while THP1 cells are already strongly affected at a low concentration. The networks show for up- and downregulation for the THP1 cells the most pathways. Additionally, the networks show gene overlap between almost all pathways for all conditions.

Conclusions: The approach allowed us to focus the analysis on affected cytotoxic processes and highlight cell-specific effects. The results showed that Caco2 cells are more resilient to TiO2-nanobelts exposure compared to SAE cells, while THP1 cells were affected the most. The automated workflow can be easily adapted using other Gene Ontology terms focusing on other biological processes.

Keywords: nanomaterials; titaniumdioxide; nanobelts; overrepresentation analysis; gene ontology; THP1; SAE; Caco2
Introduction

Engineered nanomaterials have become important in our daily life as they are utilized in the fields of food, packaging, cosmetics, drug delivery, and many others [1]. For example, silver and carbon nanotubes are used in a variety of cleansers because of their antimicrobial properties, and silicon dioxide is used as a food additive as it decreases viscosity and regulates acidity [2]. Nevertheless, some nanomaterials, such as asbestos fibres and silica dust, show how small particles can cause adverse outcomes to those exposed to them [3, 4, 5, 6].

The detailed biological processes related to the toxicity of many engineered nanomaterials are not all yet fully understood [7, 8]. Due to the varied physicochemical properties of nanomaterials and often even of the particles within the nanomaterial itself, it is difficult to predict the biological effects leading to toxicity. Biological response and toxicity depend on the size of the nanoparticle itself, size of the agglomerate, surface impurities, and degradability [9]. Furthermore, other things that have an effect on the biological response and toxicity include the way of exposure, the entry route into the human body, and the distribution in the body have an effect on the biological response and toxicity [2]. The shape of a nanoparticle also has an influence on the nanoparticle’s effects, where for example nanobelts, a nanostructure in the form of a belt, shown to be more pro-inflammatory compared to spheres [10]. Studies have found that there are various biological effects and toxicity of engineered nanomaterials under different circumstances and with varying engineered nanomaterials [1, 11, 12, 13, 14]. These changes in biological effects are ascribed to the differences in chemical and physical properties of nanomaterials. However, studying the property – biological effect relation is complicated because of the difficulty to create identical nanomaterials from different batches and/or sources due to the varying physicochemical properties of nanomaterials [15].

A relatively well-studied and widely used nanomaterial is titanium dioxide (TiO$_2$). Due to its general properties, such as the photocatalytic activity and white color, it is used in various applications such as (photo)catalysis, antibacterial agents, and consumer products. Whereas TiO$_2$ particles are shown not to be able to penetrate through the skin, entry into the body can happen via inhalation or ingestion where it then has to pass the gastrointestinal tract [16, 17].

Regarding the adverse outcomes, the toxic effects of TiO$_2$ are known to happen in order, like most toxicity related biological processes. TiO$_2$ is known to induce reactive oxygen species production which involves lipid peroxidation and eventually will lead to cell damage and DNA damage [18, 19]. Furthermore, The increase in oxidative stress can contribute to the promotion of apoptosis of the affected cells [20, 21]. Consequently, TiO$_2$ nanoparticles have been shown to induce inflammation [22, 23] among other things via the aforementioned oxidative stress in mammalian cells [18, 24]. It has been shown that TiO$_2$ nanoparticle exposure can lead to impaired immune homeostasis including increases in TNF-α, IFN-γ, IL-2, IL-4, IL-6, IL-8, and IL-10 secretion [25, 26, 27].

To study the detailed mechanisms of the cellular response, bioinformatics, and systems biology approaches, including pathway and network analysis, were used to assess the effects on toxicity-related processes upon exposure to TiO$_2$-nanobelts, i.e. reactive oxygen species formation and oxidative stress, apoptotic cell death,
inflammation, and DNA damage [28, 29]. Pathway enrichment analysis helps to put high-throughput biological data such as transcriptomics into a biological context [30] and the visual diagrams from pathway databases such as WikiPathways [31] and Reactome [32] provide a way to visualize the effects on cellular processes. However, navigating all the affected pathways and the roles of the genes in these pathways can be nontrivial: genes can participate in multiple pathways, and pathways tend to show overlap. Moreover, to combat the broad, difficult to interpret results that overrepresentation analysis and pathway visualization can yield, we need to be able to focus on detailed, molecular pathways related to higher level gene ontology processes, such as apoptosis, inflammation, DNA damage, and oxidative stress. Overrepresentation, with respect to these processes alone, makes it possible to focus on a subset of genes, but it does not have the link to the pathways. Instead, we used the enrichment of toxicity-related genes to select relevant pathways and further studied these using network analysis approaches to investigate pathway overlap and crosstalk.

In this study we re-examined a publicly available dataset generated by Tilton et al. [33] (accession number: GSE42069) [34] to study the detailed molecular mechanisms of TiO$_2$-nanobelts toxicity. We analyzed gene expression data from three different cell lines exposed to either one of two concentrations of TiO$_2$-nanobelts for 24 hours. The cell lines used were human primary small airway epithelial cells (SAE), human monocytic cells (THP1), and human epithelial colorectal adenocarcinoma cells (Caco2). These cell lines represent three common areas of exposure in the human body i.e. skin epithelium, colon epithelium, and the innate immune system that typically will respond to particles that entered the body. We aim to provide an overview of the dose-dependent and cell type-specific response by focusing on toxicity-related processes. Apart from a significant increase in the activity of toxicity-related processes after exposure with a higher dosage, differences in intensity, and affected processes might occur between the cell lines. Using this work as an example, we will discuss the toxicological effects of TiO$_2$, in particular the nanobelt shape, on the three cell lines and the importance and benefits of analysis workflows in the nanomaterial research field.

**Results and discussion**

**Differential gene expression**

The differential gene expression analysis for the three cell lines after exposure to two different TiO$_2$-nanobelt concentrations show that a high concentration of TiO$_2$-nanobelts (Figure 1, right column) causes stronger gene expression changes in all three cell lines than at a low concentration (Figure 1, left column). While the Caco2 and SAE cells also show an increased response to the high TiO$_2$-nanobelts concentration, the THP1 cells respond much stronger to the high concentration in terms of differentially expressed genes passing our criteria. Dose-dependent increases in response have been shown for many nanomaterials [35, 36].

**Pathway analysis**

Using the up- and downregulated genes from the different cell lines, overrepresentation analysis was performed to identify altered pathways after TiO$_2$-nanobelt exposure.
Figure 1 Gene expression volcano plots for different cell lines and TiO$_2$-nanobelts concentrations. On the x-axis log$_2$(fold change) is depicted whereas on the y-axis the -log$_{10}$(p-value) is depicted. The dotted lines represent cut-off values for significantly changed genes (absolute log$_2$ fold change > 0.26, p-value < 0.05). Brown color depicts that a gene meets both cut-off criteria, a blue color relates to meeting only the p-value cut-off, an orange color relates to meeting only the log2 fold change cut-off and grey color indicates that a gene does not meet any of the criteria.

The number of significantly overrepresented pathways (p-value < 0.05) are shown in Table 1 in the column “Significant”. Concordant to the increase in differentially expressed genes matching our criteria for the THP1 cell line, shown in Figure 1, the number of significantly up- and downregulated pathways increases with a surge in the concentration of TiO$_2$-nanobelts. Whereas there was also an increase, but smaller, in the number of genes found for the other two cell lines, this did not directly translate to an increase in overrepresented pathways. While over-representation analysis provides a great overview of all processes that are affected, it takes time to go manually over the many overrepresented pathways. Therefore, an automated method to select desired pathways, i.e. toxicity-related pathways, gives a new approach to interpret the data.

Toxicity-related pathways
To gain more insight into the toxicity-related processes, the altered pathways were further categorized into pathways related to apoptotic process, inflammatory response, DNA damage response, and/or oxidative stress. Often this kind
of clustering of the pathways is done manually. We implemented a gene-based approach that can automate this step. First, gene sets of four Gene Ontology (GO) terms were obtained, i.e. “apoptotic process” (GO:0006915, 1211 genes), “inflammatory response” (GO:0006954, 382 genes), “cellular response to DNA damage stimulus” (GO:0006974, 733 genes), and “cellular response to oxidative stress” (GO:0034599, 161 genes). Evidently, these processes are tightly connected. Whereas some can be causative for others, they are expected to overlap. Additional file 1 shows the gene overlap between the four GO gene sets in a Venn-diagram (see Additional file 1). Next, overrepresentation analysis identifies pathways in the WikiPathways pathway collection which are enriched for one or more of the four GO term gene sets. As an example, if a pathway is significant for an inflammatory response that means that are more inflammatory response associated genes in that pathway than you would expect for a random distribution of genes from this class over all pathways. This indicates that at least part of this pathway describes or directly links to the inflammatory response process.

Out of the 1,076 pathways in the human pathway collection, 425 pathways are affiliated to the apoptotic process, 259 are linked to oxidative stress, and 233 are linked to inflammatory response and DNA damage response as well (see Additional file 2). Interestingly, while oxidative stress is only associated with 161 genes, there are 259 pathways that are overrepresented with these genes. Some pathways focus on oxidative stress in particular (wikipathways:WP408), but cellular stress is a well-studied process and often part of other pathways like a response to caloric restriction (wikipathways:WP4191), heat stress (wikipathways:WP3395), or UV-light exposure (wikipathways:WP4482). Our analysis reconfirmed that pathway boundaries are arbitrary and there is a lot of cross-talk and therefore overlap between pathway models. That means if a pathway is enriched for a specific GO-term, it does not mean that the whole pathway describes that process, but that at least a part of the pathway directly links to the specific process of interest. By visualizing the gene overlap between the pathways associated with the different GO terms in the pathway-gene network, we can see how strongly connected and interlinked the pathways are (see Additional file 3-6).

In the last step, we filter the affected pathways in the different datasets and only keep those that are affiliated to at least one of our four toxicity related GO terms. This enabled us to perform a more focused study of the toxicity-related pathways affected under the different experimental conditions.

Study effect on toxicity-related pathways
The number of altered toxicity-related pathways is shown in Table 1. The analysis was performed separately for up- and downregulated genes. Caco2 cells show mainly downregulated toxicity-related processes in both, low (9 vs. 50) and high (11 vs. 46), concentrations which could be an indication of a very little toxic response to the TiO$_2$-nanobelts exposure. While detailed scrutiny is needed to check whether these pathways overrepresented with downregulated genes either stimulate or inhibit the process. For SAE cells, slightly more toxicity-related processes were upregulated (16) compared to downregulated (14) for the low concentration while the toxic response increased with the high concentration (49 upregulated and 22 downregulated
pathways). THP1 cells show clear toxic response activation with 36 upregulated and 6 downregulated for low concentration and 176 up- and 44 downregulated for the high concentration.

Table 1  Table depicting the number of significantly overrepresented pathways, altered toxicity pathways and the number of altered toxicity pathways for each GO-term.

| Pathways    | Significant 1,076 | Toxicity pathways 566 | Apoptosis 425 | Inflammation 233 | DNA damage 221 | Oxidative stress 259 |
|-------------|------------------|-----------------------|--------------|-----------------|----------------|---------------------|
| Caco2-low   | Up 18            | Down 53               | 4            | 0               | 9              | 1                   |
|             |                  |                       | 31           | 21              | 28             | 22                  |
| Caco2-high  | Up 16            | Down 56               | 10           | 6               | 1              | 3                   |
|             |                  |                       | 39           | 24              | 18             | 28                  |
| SAE-low     | Up 21            | Down 51               | 6            | 8               | 6              | 3                   |
|             |                  |                       | 11           | 5               | 5              | 5                   |
| SAE-high    | Up 59            | Down 44               | 41           | 32              | 14             | 32                  |
|             |                  |                       | 14           | 3               | 14             | 7                   |
| THP1-low    | Up 43            | Down 13               | 34           | 31              | 11             | 22                  |
|             |                  |                       | 6            | 3               | 3              | 2                   |
| THP1-high   | Up 197           | Down 82               | 155          | 105             | 68             | 114                 |

Importantly, while molecular pathways describe a process on a detailed level, their boundaries are often not clearly defined. Pathways are not independent, and they interact with each other through shared genes or subpathways. Figure 2 shows the gene overlap between the altered toxicity-related pathways in pathway-gene networks and highlights the differences in response between the cell lines. Pathways that cluster together indicate that these pathways depict similar biological processes with a high gene overlap. In the following sections, the biological pathways affected in the different cell lines will be discussed in detail.

Caco2 cells
Caco2 cells show immune-related processes that possess an overrepresentation of downregulated genes, such as interleukin signaling, immune system responses, and inflammation. Furthermore, for the upregulated pathways not that many pathways are significantly overrepresented after using the filtering based on the toxicity related pathways. While for the high concentration there was not a big increase in the number of significantly overrepresented pathways for the upregulated genes (9 to 11), there was even a decrease in the number of pathways for the downregulated genes (50 to 46). Similar pathways are significantly overrepresented for both concentrations. These results indicate that the studied processes in Caco2 cell lines are not extensively affected by the administration of TiO$_2$-nanobelts, for either the low or the high concentration. This finding supports the conclusion based on the cell viability assay results in the original study which showed that there was no significant decrease in cell viability for the Caco2 cell line for both concentrations of TiO$_2$-nanobelts [33].

SAE cells
Next, for the SAE cell line exposed with the low concentration, we found 16 upregulated and 14 downregulated. For the high concentration, the SAE cell line shows more affected pathways i.e. 49 upregulated and 22 downregulated compared to the low concentration. This increase in pathways with a significantly increased over-representation of affected genes is an indication that upon exposure to the high concentration of TiO$_2$-nanobelts more biological processes related to toxicity are
affected compared to the low concentration. More distinct pathways such as Oxidative Stress (wikipathways:WP408, [37]) and Apoptosis (wikipathways:WP254, [38]) are among the upregulated pathways for the high concentration while these do not show significant overrepresentation for the low concentration. The original paper showed a significant decrease in cell viability upon exposure to the high concentration of TiO$_2$-nanobelts as well [33]. In addition, pathways related to DNA damage repair are among the downregulated pathways for the high concentration. As oxidative stress is one of the major contributors to DNA damage [39] it is interesting to see that Oxidative Stress (wikipathways:WP408, [37]) is among the significantly overrepresented pathways in relation to the upregulated genes, whereas DNA damage repair pathways are among those related to the downregulated genes. This is unexpected as DNA damage and the respective repair of such damage is inherently connected to oxidative stress and apoptosis [40, 41]. However, a consequence might be that the cells give up DNA repair due to the perpetuated damage and the inevitable apoptosis. While this may seem logically, to our knowledge hard evidence is lacking.
THP1 cells
For the THP1 cell line, there are only six significantly overrepresented pathways for the low concentration of TiO$_2$-nanobelts in relation to the downregulated genes. Whereas for the upregulated genes there are 36. Among these pathways, there are numerous that are related to immune response and inflammation. This indicates that upon exposure to the low concentration of TiO$_2$-nanobelts the THP1 cell line activates immune and inflammation-related processes. This result can be explained as a general cell activity response due to the fact that THP1 is a macrophage-like cell line. However, it can also be explained due to the effect of TiO$_2$-nanobelts on the THP1 cell line in this case. TiO$_2$-nanobelts are likely to induce toxic processes, even at a low concentration, which results in an inflammatory response of the THP1 cells. Similar inflammatory responses, like activation of Nf-$\kappa$B and production of TNF-$\alpha$, were seen in these cells upon exposure to other nanoparticles such as ZnO NM-110, SiO$_2$ NM-200 and Ag NM-300 [42].

Compared to the low concentration, the high concentration yields more pathways with significant overrepresentation i.e. 176 versus 36 upregulated and 44 versus 6 downregulated. For the downregulated pathways regulatory and signaling pathways such as Wnt Signaling (wikipathways:WP428, [43]) and Post-translational protein phosphorylation (wikipathways:WP4110, [44]) pop up. Whereas for the upregulated pathways not only the immune and inflammation-related pathways pop up but also DNA damage repair pathways such as Mismatch repair (wikipathways:WP3381, [45]) and DNA IR-Double Strand Breaks (DSBs) and cellular response via ATM (wikipathways:WP3959, [46]) and Oxidative stress pathways such as Oxidative Damage (wikipathways:WP3941, [47]) and Oxidative Stress (wikipathways:WP408, [37]) pop up, compared to the low concentration. These results indicate that the THP1 cell line upon exposure to the high concentration of TiO$_2$-nanobelts results in toxicity-related processes such as inflammation, DNA damage, and oxidative stress. Which is an indication that the high concentration induces greater effects than the low concentration. This was also seen in the original paper where the low concentration induced a significant decrease in cell viability, while the high concentration induced an even greater decrease [33]. Furthermore, it has also been shown that nanoparticles can activate similar processes, such as inflammatory processes and DNA damage, as seen upon exposure to the high concentration [42, 48].

Comparison between cell lines
The focused analysis of alterations in toxicity-related processes showed strong differences between the three cell lines and the concentrations studied. It has been reported before that the molecular response depends on the cell type and concentration [49].

Caco2 cells seem very resilient to the exposure and show very little activation of toxicity-related processes. The passage of nanoparticles through the cellular barriers of Caco2 cells has been shown to be limited which could result in reduced uptake and therefore less toxic response [50]. Subsequently, this indicates a lower importance of gastro-intestinal uptake in general. On the other hand, TiO$_2$-nanobelts were already cytotoxic to THP1 cells at a low concentration, as they are more sensitive
to exposure compared to epithelial cell lines RLE-6TN and BEAS-2B [51]. Moreover, it has been reported that this response is specific to the nanobelt form of TiO$_2$ [51].

Interestingly, more than 50% of the pathways in WikiPathways and Reactome can be categorized as toxicity-related which highlights the fundamental cellular processes involved but could also indicate a bias in the pathway collections towards these well-studied processes. Nonetheless, these pathways are considered important pathways, hence they are studied well. Noticeably, after the over-representation analysis, in SAE and THP1 cells nearly all of the upregulated pathways are toxicity-related pathways supporting a clear cytotoxic response in those cells.

To illustrate how the effects can be studied in more depth we visualized the log fold change of all six conditions on the Oxidative Stress pathway (wikipathways:WP408 [37]), the Apoptosis pathway (wikipathways:WP254 [38]), the DNA Mismatch Repair pathway (wikipathways:WP531 [52]) and the Toll-like Receptor Signaling pathway (wikipathways:WP75 [53]). It shows that for most genes in these pathways gene expression data is present (see Additional file 7-9).

Interestingly, for the Oxidative Stress pathway, shown in Figure 3, the NFKB1 gene, which encodes for the Nf$\kappa$B-p105 subunit, has the highest log fold change for the THP1 cell line, high concentration. Additionally, the THP1 cell line, low concentration shows a positive log fold change as well, whereas the SAE cell line shows negative log fold changes and the Caco2 cell line noticeably lower positive log fold changes for both concentrations. Furthermore, the SOD2 gene, which is involved in the conversion of superoxide and protects against oxidative stress, shows a similar pattern [54]. These genes indicate that the THP1 cell line responds to oxidative stress by increasing the expression of protective genes.

Moreover, the Apoptosis pathway shows positive log fold changes of the CASP2 and CASP7 genes, which are involved in apoptosis execution, for the SAE and THP1 cell lines for both concentrations whereas the Caco2 cell line shows no negative or positive log fold change [55, 56]. Moreover, the pathway shows positive log fold changes for the apoptosis promoting interferon regulatory factors such as IRF4 and IRF5 [57, 58, 59], which is a similar pattern as described for the CASP2 and CASP7 genes. This could be an indication that apoptosis is stimulated in these cell lines. However, the original study by Tilton et al. does not show a significant decrease in cell viability. The DNA Mismatch Repair pathway shows a positive log fold change for the Caco2 THP1 cell lines for the LIG1 gene, which encodes for DNA ligase 1. This enzyme is involved in both DNA replication and in this context more importantly repair [60]. The aforementioned genes show positive log fold changes for the THP1 cell line throughout the pathway. The increase in expression of these genes could be an indication that DNA mismatch repair is activated due to exposure to TiO$_2$-nanobelts.

Furthermore, the Toll-like Receptor Signaling pathway showed positive log fold changes for genes such as TNF, IL8, IL1B, CCL4 and CCL5. The strongest increase in expression is seen for the THP1 cell line, whereas the SAE cell line shows increases in log fold changes as well. The Caco2 cell line, however, showed decreases in log fold change for these genes. The genes are involved in pro-inflammatory processes such as T-cell stimulation and chemotaxis [61, 62, 63]. An increase in log fold change for these genes could indicate the stimulation of an inflammatory response due to exposure to the TiO$_2$-nanobelts.
The advantage of our approach

Enrichment analysis for gene expression data is well-established and can be easily automated to increase reproducibility [64]. The interpretation of the long list of altered, often overlapping pathways is still a challenge. By providing an automated approach to filter the pathway enrichment result towards a specific biological focus, in this case toxicity, a more context-specific interpretation is facilitated. The generated pathway-gene networks showcase the connectedness of the processes and provide a more systemic view than looking at individual pathways. In addition, the approach yields a fast and easy method to select pathways of interest for more detailed scrutiny as we have shown. While interpretation and comparison between multiple datasets on a process level are still challenging, the automated analysis workflow used supports the exploration of the data [65].

Conclusion

This study investigated the molecular response of three different cell lines to exposure to TiO₂-nanobelts. Using our process-level analysis based on pathway analysis,
pathway selection using gene sets, and network visualization, our findings support the results by S.C. Tilton et al. showing that the three cell lines, Caco2, SAE, and THP1, show very different toxicity-related responses to the exposure of TiO$_2$-nanobelts from very resilient Caco2 cells to strongly responding THP1 cells. The latter is not unexpected since the observed effects align with the biological function of these immune cells. The higher effect on the lung derived SAE cells compared to the gut derived Caco2 cells suggests that further evaluation of exposure via inhalation might be relevant. Importantly, the approach allowed us to not just find changes in gene expression, but also responding molecular pathways via the pathway analysis and, additionally, allows us to filter the broad pathway enrichment results to a focused view on the cytotoxic processes affected. This allows us to visualize and explore the interactions between responding genes, based on underlying molecular processes. This versatile approach can be easily adapted to isolate other processes by using other Gene Ontology terms diverting the focus to other biological processes of interest.

Materials and methods

Dataset
In this study, a published and publicly available transcriptomics dataset generated by Tilton et al. [33] was used. The dataset is available from the Gene Expression Omnibus (accession number: GSE42069) [34]. Quality control, data pre-processing, and statistical analysis were performed using scripts from ArrayAnalysis.org [66].

The dataset consisted of 18 samples from three human-like cell lines, i.e. Caco2, SAE, and THP1, which were exposed to either medium (control), 10 µg/ml or 100 µg/ml TiO$_2$-nanobelts for 24 hours in triplicate. Culture conditions were kept as identical as possible between the three cell lines. Details can be found via the original publication by Tilton et al. [33].

Volcano plots depicting differential gene expression were made using Enhanced-Volcano package (version 1.4.0) for R (version 3.6.1) [67, 68]. Genes were considered differentially expressed between treated and control when they had an absolute log2 fold change greater than 1.2 and a p-value lower than 0.05.

Pathway analysis
Overrepresentation analysis was performed using the enricher function of the clusterProfiler package (version 3.14.3) [64] for R to identify the molecular changes on a pathway level. The human pathway collection containing 1076 pathways was obtained from WikiPathways (http://www.wikipathways.org, version 20201003, [31]). The curated and Reactome collections from WikiPathways were included in the analysis. Enrichment analysis was performed separately for up- and downregulated genes using the fold change and p-value cutoff as described in the previous section. Additionally, default settings of the enricher function of the clusterProfiler package were used except p-value and q-value (false discovery rate) cutoff were set to 1 to include all results at this stage. This allows us to later select the desired results based on a p-value smaller than 0.05. Relatively small and very big pathways were omitted from the analysis by setting the minimal gene set size to 10 and the maximum gene set size set to 300. Results were visualized as pathway-gene networks.
Toxicity-related processes
Within the two human pathway collections used, toxicity-related pathways were identified based on the enrichment of toxicity-related gene sets using the clusterProfiler R package. The gene sets were retrieved from the Gene Ontology (GO, version: release 2020-06) for the following four toxicity-related GO-terms: “apoptotic process” (GO:0006915), “inflammatory response” (GO:0006954), “cellular response to DNA damage stimulus” (GO:0006974) and “cellular response to oxidative stress” (GO:0034599) [69, 70]. Associated genes were retrieved using the biomaRt package in R (version 2.42.0, Ensembl Genes 100) [71, 72]. Using the GO evidence codes, only genes with experimental evidence or manually curated annotations were included to ensure high confidence that the gene is involved in the specific process (IBA, IC, IDA, IEP, IGI, IMP, IPI, TAS, http://geneontology.org/docs/guide-go-evidence-codes/, see Additional file 10). Gene overlap between GO-terms was visualized using Venny version 2.1.0 [73].

For the enrichment analysis, the same settings were used as with the pathway analysis described above.

Network visualization
Altered pathways in the enrichment analysis were filtered for toxicity-related pathways and then visualized as pathway-gene networks to portray the overlap and crosstalk between the pathways. To construct the networks, edge (source: pathway, target: gene) and node (all unique source and target nodes) files were created of the altered pathways. The networks were made using the igraph R package (version 1.2.4.1) [74].

Reproducible analysis workflow
The complete analysis is automated in R (version 3.6.3) and can be easily repeated with a new transcriptomics dataset or different selection focus. The R scripts are available on GitHub (https://github.com/laurent2207/TiO2-scripts) and archived on Zenodo [65].

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Availability of data and material
The R-markdown scripts and corresponding files are available via Zenodo: [https://doi.org/10.5281/zenodo.4569568] [65], where the GitHub repository is available under MIT license [https://github.com/laurent2207/TiO2-scripts/blob/master/LICENSE].

Competing interests
The authors declare no conflict of interest.

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Author’s contributions
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Additional Files

Additional file 1 — Venn diagram showing the number of overlapping genes between the four GO-terms. Venn diagram showing the number of overlapping genes between the four GO-terms: “apoptotic process”, “inflammation”, “cellular response to DNA damage stimulus”, “DNA damage and Oxidative stress”.

Additional file 2 — Number of significant pathways (p < 0.05) and number of genes per Gene Ontology category. Number of significant pathways (p < 0.05) and number of genes per Gene Ontology category.

Additional file 3 — Overrepresentation analysis results of “cellular response to oxidative stress”. Network which shows pathway-gene-pathway interactions. Grey node represents a gene, the light brown node represents a pathway. Size depicts gene count. Pathways show genetic overlap and no separate clusters, which is an indication of these pathways being related to similar biological processes.

Additional file 4 — Overrepresentation analysis results of “apoptotic process”. Network which shows pathway-gene-pathway interactions. Grey nodes represent genes, the light brown node represents a pathway. Size depicts gene count. Pathways show genetic overlap and no separate clusters, which is an indication of these pathways being related to similar biological processes.

Additional file 5 — Overrepresentation analysis results of “inflammatory response”. Network which shows pathway-gene-pathway interactions. Grey node represents a gene, the light brown node represents a pathway. Size depicts gene count. Pathways show genetic overlap and no separate clusters, which is an indication of these pathways being related to similar biological processes.

Additional file 6 — Overrepresentation analysis results of “cellular response to DNA damage stimulus”. Network which shows pathway-gene-pathway interactions. Grey node represents a gene, the light brown node represents a pathway. Size depicts gene count. Pathways show genetic overlap and no separate clusters, which is an indication of these pathways being related to similar biological processes.

Additional file 7 — Visualization of log fold change on the Apoptosis pathway (wikipathways:WP254) for all six conditions. Cell lines are depicted from left to right as Caco2, SAE and THP1. The top row depicts the low concentration whereas the bottom row depicts the high concentration. Gradient goes from blue (log fold change < -0.58) via white (log fold change = 0.0) to red (log fold change > 0.58).

Additional file 8 — Visualization of log fold change on the DNA Mismatch Repair pathway (wikipathways:WP531) for all six conditions. Cell lines are depicted from left to right as Caco2, SAE and THP1. The top row depicts the low concentration whereas the bottom row depicts the high concentration. Gradient goes from blue (log fold change < -0.58) via white (log fold change = 0.0) to red (log fold change > 0.58).

Additional file 9 — Visualization of log fold change on the Toll-like Receptor Signaling pathway (wikipathways:WP75) for all six conditions. Cell lines are depicted from left to right as Caco2, SAE and THP1. The top row depicts the low concentration whereas the bottom row depicts the high concentration. Gradient goes from blue (log fold change < -0.58) via white (log fold change = 0.0) to red (log fold change > 0.58).

Additional file 10 — List of Gene Ontology (GO) evidence annotation codes. List of Gene Ontology (GO) evidence annotation codes which were used to remove genes related to the four GO-terms with these annotations (bottom part). Additionally, the annotation codes that were present are shown (top part).