Dynamics and regulation of the oxidative stress response upon chemical exposure

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General discussion and future prospects

As we are constantly exposed to a broad spectrum of chemicals, which may lead to adverse health effects, developing human relevant mechanistic biomarkers which provide information regarding the type of exposures and its (predicted) effect are from utmost importance.

THE EVOLUTION OF BIOMARKERS: FROM A SINGLE MOLECULE TOWARDS A BIOMARKER FINGERPRINT

As described in **Chapter 1**, the perspective of a biomarker is changing over time. This process is driven by new technologies enabling us to measure a wide spectrum of, for example, genes, proteins and metabolites in a short amount of time as well as increased effort to reduce the number of animal tests. Moreover, techniques like for example high content high throughput microscopy in combination with GFP-reporter cell lines makes it possible to study biological responses over time, providing us valuable information concerning the onset, magnitude and progression of the stress response pathway. Furthermore, spatial-temporal analysis provide information regarding the place and time of the molecules in the signal transduction cascade that is specific for a type of chemical stress. The large amount of data created by these techniques enables us to search for the interactions in biological processes which in turn provides opportunities for biological network analysis. Biological network analysis, like for example gene-gene interaction networks, co-regulation / co-expression networks, Bayesian networks, and weighted gene co-expression analysis (WGCNA) makes it possible to enhance our understanding of these molecular interactions and to identify the molecules which play a central role in a biological network (Charitou et al. 2016; Saelens et al. 2018), and therefore may be promising candidate biomarkers of chemical exposure and disease. Moreover, WGCNA approaches combining gene expression data with histology and clinical chemistry data can be used to predict adverse and non-adverse outcomes of chemical exposure and allows translation from animal to human (Callegaro et al. 2021; Sutherland et al. 2018). Recently, WGCNA was also conducted on microRNA data to find new biomarkers (Qin et al. 2019; Soleimani Zakeri et al. 2020). Interestingly, integrating microRNA data (microRNA and mRNA co-expression modules) also makes it possible to examine the regulatory roles that microRNAs have on their target genes (Mamdani et al. 2015). Therefore, including microRNA data into WGCNA modules show great promise, making it possible to obtain a more comprehensive coverage to elucidate the underlying mechanisms of toxicity as cannot be achieved by using gene expression data as the only source of data (Ma et al. 2019).

MICRORNAS AS BIOMARKERS OF EXPOSURE AND DISEASE

As described above, microRNAs are new players which might be part of a biological fingerprint. In **Chapter 2**, the use of microRNAs as biomarkers of chemical exposure and disease are described. Interestingly, we found several microRNAs to be differently expressed after exposure to different chemicals, like for example miR-21 and miR-26. Of course a change in, for example, miR-21 does not provide any information about a change in health state. However, we must be careful to link a change in expression of only one or two microRNAs to a particular chemical exposure or effect. Most of the biomarkers identified in the various studies have analyzed microRNA changes in the cells, either in cell culture *in vitro* or tissue i*n vivo*. It remains unclear whether these microRNAs can be detected in the blood and thereby represent a mechanistic biomarker that could reflect on the mode of action of a particular chemical exposure. So far biomarkers measured in the blood have not per se been discovered in relation to their mode of action. Therefore, the question still has to be answered whether or not the rise of certain microRNAs found in the blood has indeed a functional role, or that the change in expression is just the result of tissue damage. However, microRNAs which are known to be strongly related to a specific organ, like miR-122 for the liver, can provide valuable information about which organ is damaged upon exposure to a chemical or during disease, as miR-122 makes up for 70% of the total pool of microRNAs in the liver (Jopling 2012) and is linked to e.g. cholesterol/lipid metabolism, iron homeostasis, and differentiation of hepatocytes. However, the rise of miR-122 levels measured in blood on its own has no relation to the type of toxic liver injury (Madboly et al. 2019). Interestingly, hepatocellular carcinoma cells, like HepG2 cells, have lower levels of miR-122 as compared to normal liver cells. The loss of miR-122 is related to downregulation of tumor cell apoptosis, hepatic cell invasion, interhepatic metastasis and reduced sensitivity towards drugs (Ha et al. 2019; Xing et al. 2013) making miR-122 restoration as a treatment interesting for the clinic (Ha et al. 2019). Defining whether the candidate microRNA biomarkers play an integral functional role in disease mechanism is a difficult task. One single microRNA might have hundreds of different targets and the role of the microRNA might differ between different tissues. Furthermore, a microRNA can both increase or diminish the stress response depending on their involvement in the inhibition of negative regulators in a positive feedback loop or as part of a negative feedback loop, respectively (Emde and Hornstein 2014).

New emerging techniques to measure microRNAs in *in vitro* and *ex-vivo* derived biological samples, like for example droplet digital PCR, makes it possible to quantify microRNA copy number of multiple microRNAs in a single sample (Stein et al. 2017). Moreover, the measurement of microRNAs derived from extracellular vesicles (EVs) is

growing in popularity as EVs might in some cases provide a more consistent source of microRNAs compared to circulating-free microRNAs associated with proteins (Endzelins et al. 2017). However, robust and high throughput isolation of specific groups of EVs is still challenging (Buschmann et al. 2018).

MIMICKING THE OVEREXPRESSION OF CERTAIN MICRORNAS CAN ENHANCE OR INHIBIT THE SRXN1 RESPONSE IN HEPG2 CELLS

Investigating the role of microRNAs in a stress response pathway might be accomplished by using microRNA inhibitors or mimics. In **Chapter 3**, research is presented making use of microRNA mimics, so mimicking overexpression of a particular microRNA in a cell. Making use of different HepG2-GFP reporter cells in combination with live cell confocal microscopy, we were able to study the effect of almost all individual microRNA directly on GFP-reporter protein level at the single cell level. We identified several microRNAs which, when overexpressed, are able to enhance or reduce the expression of sulfiredoxin (Srxn1), a sensitive biomarker for the induction of oxidative stress that is a direct downstream target of the transcription factor Nrf2 (Soriano et al. 2008). The same pattern was observed in gene expression changes of sulfiredoxin. However, the microRNAs found to alter the expression of sulfiredoxin in this study, are not selective for the Nrf2 pathway as they were also able to regulate the expression of other genes that are part of other (stress response) pathways, such as the unfolded protein response. Of course this might be explained by the fact that those microRNAs have hundreds of different targets. However, we also have to keep in mind that most stress-response pathways are linked to each other (Bhattarai et al. 2021) and that, *in vivo,* multiple different microRNAs can "work together" by targeting the same gene, where the change in expression is the resultant of all the different microRNAs (Peter 2010). Therefore, to obtain information concerning the primary response upon microRNA expression changes, there is a need to perform further temporal analysis of the transcriptional changes after microRNA transfection. High throughput transcriptomics based on TempO-seq is a preferred cost effective method for this.

Interestingly, some of our most potent microRNA candidates would have been missed by various online target prediction tools. This suggests the limitations in the prediction of these *in silico* tools and indicates that biological microRNA screens as performed during our studies is of high importance. The microRNAs found in

our study that enhance or inhibit the Nrf2 pathway might provide opportunities for microRNA therapeutics that target the Nrf2 pathway. Enhanced Nrf2 pathway activation is considered as a pro-oncogenic pathway. This is exemplified by the high penetrance of *KEAP1* mutations in e.g. lung cancer that prohibit Nrf2 ubiquitination and enhance an antioxidant stress response and resistance to anticancer therapy (Jaramillo and Zhang 2013). However, findings indicate that not changes in the genes e.g. somatic mutations in either *KEAP1* and/or *NFE2L2* itself are responsible for high Nrf2 activity, but are rather the consequence of deregulation of the transcription of Nrf2 by epigenetic factors like hypermethylation of the *KEAP1* promoter and microRNAs linked to the cell-detoxifying network (Fabrizio et al. 2018; Shah et al. 2013). Therefore, microRNAs which are able to inhibit the Nrf2 pathway might be used to make cancer cells more vulnerable for chemo- and radiotherapy. Furthermore, oxidative stress plays a central role in various acute and chronic pathologies including ischemia/reperfusion injury and neurodegenerative diseases (Arshad et al. 2017). Therefore, microRNAs enhancing the Nrf2 pathway might be used to enhance a person's protection against oxidative stress. As a consequence, drug delivery strategies to target microRNAs to specific target tissues are currently being development (Vassalle et al. 2020), providing improved strategies for Nrf2-modulating therapeutic approaches through microRNAs. Additional research on the dose response and long term safety profile of our candidate Nrf2 modulating microRNAs is required.

Caution however has to be made to link microRNAs which alter the Nrf2 response directly to Nrf2. As described by Ashrafizadeh et al. 2020, microRNAs can regulate the Nrf2 pathway via different ways: 1) by affecting the nuclear translocation of Nrf2, 2) by influencing the expression of Nrf2, regulating the upstream mediators of Nrf2 and modulation of *KEAP1* (Ashrafizadeh et al. 2020). Interestingly, also redox stress itself can alter the microRNA biogenesis and processing pathway leading, for example, to altered redox signaling an disease mechanisms (Cheng et al. 2013). Furthermore, Mendell and Olson describe five different mechanisms by which microRNAs can regulate signaling pathways in general, depending on the cellular and functional context, as a single microRNA is not limited to one of these mechanisms: stress signal mediation, stress signal modulation, negative feedback, positive feedback, and buffering (Mendell and Olson 2012).

CELLS PREVIOUSLY EXPOSED TO PRO-OXIDANTS EXHIBIT AN ALTERED RESPONSE PATTERN COMPARED TO 'NAIVE' CELLS

Biology is equipped with adaptive responses that allow cells and tissues to cope with altering environmental conditions, such as exposure to toxic substances. This also holds true for cellular stress response programs, including the Nrf2 pathway. Therefore, we studied the dynamics of the Nrf2 pathway over time and were wondering what the effect would be of a second exposure given at different stages of the response. In **Chapter 4**, we describe the outcome of this study. Interestingly, we found that the Nrf2 response after a second treatment after 24 h, was lower than the response to the first exposure with the same concentration, indicating that the Nrf2 is adaptive. However, sulfiredoxin, a downstream target of Nrf2, showed a three-fold higher response compared to the first treatment, with all cells participating in the response. Although more research is needed to unravel the precise mechanism, it is clear that repeated exposure testing will add valuable information in testing the safety of a chemical or drug. As indicated in **Chapter 4**, several aspects are important to consider in future research. First of all characteristics of the chemical compound used to induce the Nrf2 pathway, like mode of action and half-live, as these aspects determine the speed and duration of the response. Other molecules playing a role in the modulation of Nrf2 transcriptional activity might be incorporated in the study as well as other downstream targets which might behave differently upon repeated chemical exposure (Bergström et al. 2011; Mathew et al. 2014). siRNA-mediated knockdown of single or combinations of Nrf2 pathway-related molecules can be used to elucidate their role in the first and second response. Also the time between the first and second exposure can be changed, however, *in vitro,* caution has to be taken not to induce cytotoxicity. Moreover, the role of microRNAs have to be investigated in this matter as they may play a role in the induction of the second response as they are able to block the expression of genes playing a role in controlling, e.g. inhibiting the Nrf2 response (see **Chapter 3**). As indicated by several studies, preconditioning might be used for therapeutic approaches using low non-toxic concentrations (Mathew et al. 2014). However, challenge remains in finding the optimum dosing regimen: dose per treatment, time between treatments and number of repeated treatments.

TESTING STRATEGIES FOR CELLULAR STRESS RESPONSE ACTIVATION

As activation of the Nrf2 pathway upon chemical exposure might indicate oxidative damage, we hypothesized that our high throughput microscopy reporter and transcriptomics toolbox might be suitable to characterize the ability of chemicals to cause oxidative stress and activation of the Nrf2 pathway. To test this hypothesis, a panel of different phenolic compounds was used that were either redox cycling phenols, alkylated phenols, and non-redox cycling phenols. Outcomes of this study are described in **Chapter 5**. Interestingly we were able to discriminate between redox-cyclers that induce the oxidative stress response and non-redox-cyclers that lack this ability, at least at lower concentrations. Moreover, although the concentration of a compound needed to induce the Nrf2 pathway was different in HepG2 cells compared to primary hepatocytes, we were still able to discriminate between these two compound classes. This indicates that the onset of downstream targets like sulfiredoxin can indeed provide information concerning the mode of action of a compound.

Besides *SRXN1*, we also found *AKR1B10* to be a sensitive marker to discriminate between redox-cyclers and non-redoxcyclers. The Nrf2 pathway is known to be one of the major regulatory systems for *AKR1B10* gene regulation (Endo et al. 2021; Rooney et al. 2020) and therefore it was not a surprise that we found *AKR1B10* to be induced by our set of oxidative stress inducing compounds. However, we have to keep in mind that activation of the Nrf2 pathway, or a stress response pathway in general, is not a direct indication for an adverse outcome leading towards toxicity, although a harmful event is needed to activate the stress response pathway. Activation, as a consequence, may lead towards cellular protection against more harmful stimuli, as protection due to induction of a stress response pathway is not specific for the event which activated the pathway. For example, sulforaphane, a known inducer of the Nrf2 pathway present in e.g. broccoli sprouts, was found to provide cellular protection against radiation (Mathew et al. 2014). The same of course is true for phenolic compounds as used in **Chapter 5** which were chosen based on knowledge obtained in animal studies and compound structure similarity. Furthermore, Castañeda-Arriaga et al. 2018, identified the presence of redox metals, the pH, and the possibility of the formation of benzoquinones as key aspects regarding the pro- versus anti-oxidant effects of phenolic compounds and therefore these aspects have to be taken into account future research (Castañeda-Arriaga et al. 2018).

A parameter often used in toxicity testing is the point of departure (PoD) which is defined as the lowest concentration at which a response can be detected. As *SRXN1* and *AKR1B10* were found to be the most responsive, they also had the lowest PoD (Hatherell et al. 2020). Care should be taking when a marker is too sensitive, since the potential of a compound to induce for example the Nrf2 pathway and results in adversity might be overestimated. An early response might be linked to the primary mode-of-action of the compound and be indicative that targeting the Nrf2 pathway is the primary event of the exposure at low concentrations, but not part of a general toxicity response that may involve other pathways and seen at high concentrations. Therefore, activation of protective pathways at low concentrations would be indicative of a beneficial effect of the compound *in vivo* rather than an adverse health effect. As a consequence, the use of a single marker or only stress-response pathway information is not enough to fully predict the ultimate adverse effect of a chemical. For example, some organophosphate (OP) pesticides we tested for Nrf2 pathway activation potential, did only activate the Nrf2 pathway at very high concentrations; yet at lower concentrations, the toxic effect of these compounds, acetylcholinesterase inhibition, might already occur *in vivo*. As the liver is the most prominent target tissue for adverse drug reactions, most test systems used in drug safety evaluation are focused on the liver to assess novel chemical drug entities for the liability to induce drug-induced liver injury. In other areas, especially in the field of environmental safety testing, efforts are made to establish test systems to measure the toxicity of a chemical compound in multiple organs like multiorgan-on-a-chip (multi-OoC) platforms as reviewed by Picollet-D'hahan et al. (Picollet-D'hahan et al. 2021).

FUTURE PROSPECTS

As technical abilities and knowledge regarding stress response pathways is growing, future biomarkers will probably consist of different key-players of these pathways. These biomarkers will be proteins, genes, microRNAs as well as several combinations of these markers together forming a biomarker fingerprint. Combining these markers enables us to make use of the strengths of each of these molecules and to overcome the weaknesses they have when used as a single biomarker. Recent advancement in measurement technologies enables the simultaneous detection of combinations of small molecules, proteins and microRNAs in one sample. Wang and Walt (2020), for example describe the simultaneous detection of interleukin 6 and miR-141 making use of single molecule arrays (Simoa). In this assay, Dye-encoded beads modified with specific capture probes were used to quantify each analyte (Wang and Walt 2020). These multiplex detection methods make it possible to measure multiple

markers in a single assay and therefore decrease the amount of sample needed (Cai et al. 2021; Jet et al. 2021; Nagarajan et al. 2020). Therefore, combining multiplex assays combined with e.g. lateral-flow immunoassays, makes these tools suited for point of care testing in clinical settings (Huang et al. 2020).

The greatest challenge however will be the usability/accessibility of the biomarker fingerprint in the *in vivo* situation. Biomarkers intended to be used as biomarkers for disease or biomarkers of exposure established *in vitro* should preferably have applicability *in vivo*. Therefore, all members of the biomarker panel should be easily obtained, preferable in blood, urine or saliva. Second, the marker should have the same function *in vivo* as established *in vitro*. This is especially challenging for microRNAs, as microRNAs might have different functions in different tissues. Subsequently, a microRNA mechanistically linked to a stress response pathway *in vitro* cannot necessarily be linked to its established mechanistic link to the stress response pathway *in vivo*. Therefore, simultaneous detection with other biomarkers that are reflective of the same biological perturbation might underpin the *in vivo* function of the microRNA. At this stage more research is necessary to obtain information regarding the different functions of microRNAs in different settings of disease and chemical exposure.

Recently, CRISPR-Cas9 based techniques are used to aid microRNA research. A CRISPR-Cas9 based "stoplight" reporter system is described by de Jong et al. (2020), which allows direct functional study of EV-mediated transfer of small noncoding RNA molecules at single-cell resolution. Data obtained can contribute to increase our understanding of the regulatory pathways that dictate the underlying processes by which microRNA function (de Jong et al. 2020). Furthermore, Wang et al. (2019) created a miRNA sensor that can measure microRNA activity at cellular levels by using a microRNA-mediated single guide RNA (sgRNA)-releasing strategy, which can be used to monitor the differentiation status of stem cells (Wang et al. 2019). Altogether this indicates the wide use of microRNAs and the new possibilities of combining microRNA knowledge with novel state of the art technologies, like CRISPR-Cas9, in different fields of research. In turn this could pave the way towards an improved understanding to discover novel mechanistic microRNA biomarkers of chemical exposure as well as novel disease modifying biomarkers.

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