**Twenty years of proteomics in radiation biology – a look back**

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**Abstract**

**Purpose:** The aim of this article is to describe the technical development in proteomics during the last two decades with the focus on its use in radiation biology. It is written from a subjective point of view and aims not to be a scientific review of the subject.

**Conclusion:** Proteomics is a fast developing technique and it has already contributed greatly to our understanding of biological mechanisms following radiation exposure. Novel proteomics approaches can be used in adequately designed cellular and animal experiments and above all in big clinical trials to investigate effects of ionizing radiation in the future.

In the beginning of this millennium the proteomics technologies were relatively new, the enthusiasm big and the hopes high. In those days the proteomics world was split into two camps, those who preferred protein electrophoresis, particularly two-dimensional (2D) gel electrophoresis, for the protein separation, and those who were for direct identification of digested peptides using liquid chromatography coupled to tandem mass spectrometry (MS/MS), the so called shotgun proteomics. Interestingly, these two camps were also geographically separated, the Europeans favoring the gel-based method and the Americans the gel-free method. Particularly the Germans were advanced users of the 2D gels, presenting the biggest gels and the highest number of protein spots (Klose 1999; Challapalli et al. 2004).

The gel-based separation of proteins was as such not a new method. I remember running the so called O´Farrell gels during my PhD in Sweden in early 1980´s but after moving to Germany for my post-doc I realized that this name for 2D-SDS-PAGE gels was not popular there. Actually, O´Farrell and Klose discovered and published this method at the same time in the spring of 1975 (Klose 1975; O'Farrell 1975). The problem with those first gels was that one could discover changes in the protein expression or even mutations by looking at the gels (you had to rely on your eyes since there was no software available at that time) but it was not possible to identify the proteins. Edman degradation that was mainly used for purified proteins was tedious and slow. First the developments of mass spectrometry technologies such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) meant a breakthrough. By these methods one could identify the peptides (and so the proteins they originate from) by measuring the velocity of the peptide ions in a magnetic field that in its turn depended on the mass-to-charge ratio, the so called time-of-flight (TOF).

After moving to Munich and starting to work at the Federal Office for Radiation Protection I was happy to find a fully equipped laboratory for running 2D gels for proteome investigation. The colleagues there were at that time busy studying the Wismut cohort, uranium miners in East Germany during the time of the cold war who were exposed not only to ionizing radiation but also to other health hazards such as asbestos and arsenic. We decided to study the effects of radiation and arsenic on a human lymphoblast cell line, alone or in combination, using the 2D proteomics of that time. We published this study in March 2005, probably representing the first proteomics paper in radiation biology (Tapio et al. 2005). In this first paper we were able to identify seven proteins with significantly altered expression in any of the conditions.

After starting my own proteomics group at Helmholtz Center Munich in 2007, the 2D gels were still the method of the choice. Similar to the first paper the gels were digitally imaged by automatic spot detection and analyzed using suitable 2D software. We had, however, moved away from the silver staining of the gels to use fluorescent dyes, the so called 2D-DIGE technology, which significantly increased the reproducibility. By these means we were able to discover changes in the proteome of endothelial cells even at relatively low gamma doses of 0.2 Gy (Pluder et al. 2011). Increasing the dose rate seemed to increase the number of differentially regulated proteins and altogether we could identify 15 proteins.

This was interesting since more and more epidemiological data had started to emerge challenging the myth of the heart as a radiation-resistant organ and particularly clinical exposure in radiation therapy increased the risk of cardiovascular disease significantly (Little et al. 2008). My proteomics group received EU funding for two projects to investigate the biological background this increased risk. Both projects aimed to identify radiation-associated changes in the heart proteome.

In 2009 I had the fortune to employ two post-docs both possessing a recent doctoral degree from two German universities steeped in tradition, Marburg and Tübingen, respectively. We irradiated a number of mice with a range of doses and it was fun to observe the friendly but determined weekly competition of the best 2D gels and the largest number of protein spots on them. The competition was by no means fair, though, and this was due to the material that was used.

In the STORE project we had to use formalin-fixed paraffin-embedded (FFPE) material that is the main type of material in clinical but also in radiobiological archives (Tapio and Atkinson 2008). Due to the tissue preservation it was a headache to encrypt its proteome. In a way this already heralded the end of 2D gels in my lab because the gel-based approach just did not work with FFPE material. Finally, we managed to publish a really nice and well-cited paper using a comparison of gel-based and gel-free methods (Figure 1) (Azimzadeh et al. 2010). We could show that cardiac mitochondria were an important radiation target in the heart.

In the second project, CARDIORISK, we decided to isolate the mitochondria from the irradiated and control hearts and look at the changes four and forty week after the local X-ray radiation. Again, we could show the important role of mitochondria in the radiation response of the heart. The mitochondrial complexes of the electron transport chain lost their activity, less oxygen was consumed and more reactive oxygen species were produced (Barjaktarovic et al. 2011; Barjaktarovic et al. 2013). As in the FFPE paper, we used in this study both gel-based and gel-free methods. For the latter method we used isotope coded protein labeling (ICPL) combined with one-dimensional gel electrophoresis for quantification. Since then, the ICPL labeling was the “working horse” for years to come in our proteomics lab. For example, we used it successfully to study premature aging in endothelial cells triggered by chronic low-dose-rate radiation (Yentrapalli, Azimzadeh, Barjaktarovic, et al. 2013; Yentrapalli, Azimzadeh, Sriharshan, et al. 2013).

Labeling was really a hot topic in those days. Tagging the proteins or peptides with different labels enabled the quantification of proteins originating from different groups, for example mice or cells irradiated with different radiation doses or dose rates and then comparing them to the respective sham-irradiated groups. The best and unfortunately also the most expensive labeling method was Stable Isotope Labeling by Amino acids in Cell culture, SILAC. While dreaming of the “SILAC mouse” we decided to continue the study of radiation-induced alterations in endothelial cells, this time using SILAC labeling and concentrating on the immediate protein expression changes 4 and 24 hours after irradiation (Sriharshan et al. 2012). Four hours may not seem very “immediate” for scientists using millisecond timescales but this is the approximate time needed for *de novo* protein synthesis in cells. With SILAC we could identify around 3,000 different endothelial proteins, a record that held until recently. Alongside the SILAC method we still used the 2D gel electrophoresis but this paper was the last one using this approach and we left this tedious method requiring great handicraft skills feeling a sort of sadness. However, moving away from the 2D gel system resulted in a huge increase in the number of identified and quantified proteins thereby enabling us to face totally new challenges.

These came in the form of two EURATOM-financed projects, PROCARDIO and CEREBRAD, both of which aimed to investigate low-dose radiation effects in the heart and brain of postnatal mice, respectively. Interestingly, in adult mice that were irradiated ten days after the birth with doses ranging from 0.1 to 1.0 Gy the transcription factor PPAR alpha was activated in the heart (Bakshi et al. 2013). This was surprising since we had shown just the opposite, namely inactivation of PPAR alpha, in mice irradiated at a much higher local heart dose (16 Gy) in the early adulthood and also this effect was permanent (Azimzadeh et al. 2013). Indeed, an active PPAR alpha is absolutely necessary for a proper heart function since it is anti-inflammatory and even more importantly it regulates the cardiac lipid metabolism. We suggested that this transcriptional regulator is essential in pre- and postnatal phases of cardiac function and its activation is a regenerative adaptation to the initial radiation damage. The central role of PPAR alpha as a radiation target has been one of the main topics in my lab ever since.

The neonatal brain, particularly the hippocampus, seemed to be even more sensitive to radiation than the neonatal heart. The changes in the proteome were immediate and long-lasting and were coupled to defects in cognition (Kempf, Buratovic, et al. 2014; Kempf, Casciati, et al. 2014). Interestingly, the respiratory capacity of mitochondria isolated from hippocampus was significantly decreased already at 0.5 Gy four weeks after irradiation and this effect was absolutely dose-dependent (Kempf et al. 2015). We could also show that a chronic exposure to low-dose-rate irradiation resulted in changes in murine hippocampus that resembled early Alzheimer´s pathology in human (Kempf et al. 2016). In this cooperative paper with the Larsen lab we were even able to measure post-translational protein modifications arising in the hippocampus during the long radiation exposure of 300 days (Kempf et al. 2016). The age at exposure seemed to be the most important factor: the younger the age when irradiated the greater the damage (Casciati et al. 2016).

By the end of PROCARDIO and CEREBRAD where we had successfully used the ICPL labeling, sometimes with four different stable isotopes in the same experiment, we step by step left the era of labeling proteomics. The reason for this was mainly that using ICPL and most other labels only lysine-containing peptides and protein N termini could be used for quantitation, thereby leaving a small but possibly important fraction of proteins undetected.

In the years 2015 to 2017 we used ICPL and label-free methods side by side to investigate especially the radiation response in endothelial cells. For endothelial cells isolated directly from the irradiated mouse heart we still used ICPL (Azimzadeh et al. 2015) but for the acetylation studies of endothelial cells the label-free method was more suitable (Barjaktarovic et al. 2015; Barjaktarovic et al. 2017). Finally, when we received particularly valuable human material, the cardiac left ventricle autopsies from the Mayak workers, the label-free technique was the method of choice (Azimzadeh et al. 2017). The Mayak paper and the following validation (Papiez et al. 2018) confirmed that PPAR signaling but also TCA cycle and glycolysis/gluconeogenesis processes were the most important signaling pathways influenced by radiation in the heart.

We also continued the low-dose studies on the hippocampus, now reducing the dose but expanding the follow up to two years (Hladik et al. 2020). Using a mouse model we could show that already radiation doses lower than 0.1 Gy showed permanent but more or less protective anti-apoptotic and anti-inflammatory effect on the hippocampus and by increasing the radiation dose the effects became more and more adverse. At the dose of 0.5 Gy the effect was clearly negative. This was in agreement with our previous cognition studies (Kempf, Casciati, et al. 2014) and since then solidly confirmed by recent studies (Ung et al. 2021).

The latest development in our proteomics lab has been on the level of creating and analyzing the mass spectrometry data by moving from the data-dependent acquisition (DDA) mode to data-independent acquisition (DIA) mode. In DIA, only the most intense peptide ions in the first stage of MS/MS are selected to be fragmented in the second stage. In contrast, the DDA mode systematically collects MS/MS data from every mass and from all detected precursors. This enables the identification and quantification of proteins to identify and quantify samples even in complex mixtures in a large dynamic range reproducibly. We have used DIA recently to study radiation response in lung and coronary artery endothelial cells (Philipp, Le Gleut, et al. 2020; Philipp, Sievert, et al. 2020). With DIA, we could identify more than 4,000 different endothelial proteins in total, finally breaking the record from the SILAC experiments. It is also nice to note that in comparison to our first low-dose endothelial paper we could identify and quantify six times more differentially regulated proteins under comparable irradiation conditions than ten years ago (Pluder et al. 2011; Philipp, Le Gleut, et al. 2020).

This decade is bringing new possibilities to use the proteomics platform even in radiation biology. There is a trend to initiate big clinical studies for collecting easy-to-access biofluids such as plasma or serum that can be used to find radiation biomarkers, even with the help of proteomics technologies. Alongside intelligent study designs I see more than anything the improvements in novel technical solutions as promising. Especially data analysis is developing with a breathtaking speed. Cloud computing, software containers and the possibility to access excellent data banks and perform state-of-the-art data mining independent on your location or technical infrastructures available opens enormous opportunities. The future of proteomics in radiation biology will stay challenging, interesting and full of excitement!

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**Disclosure statement**

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**Biographical note**

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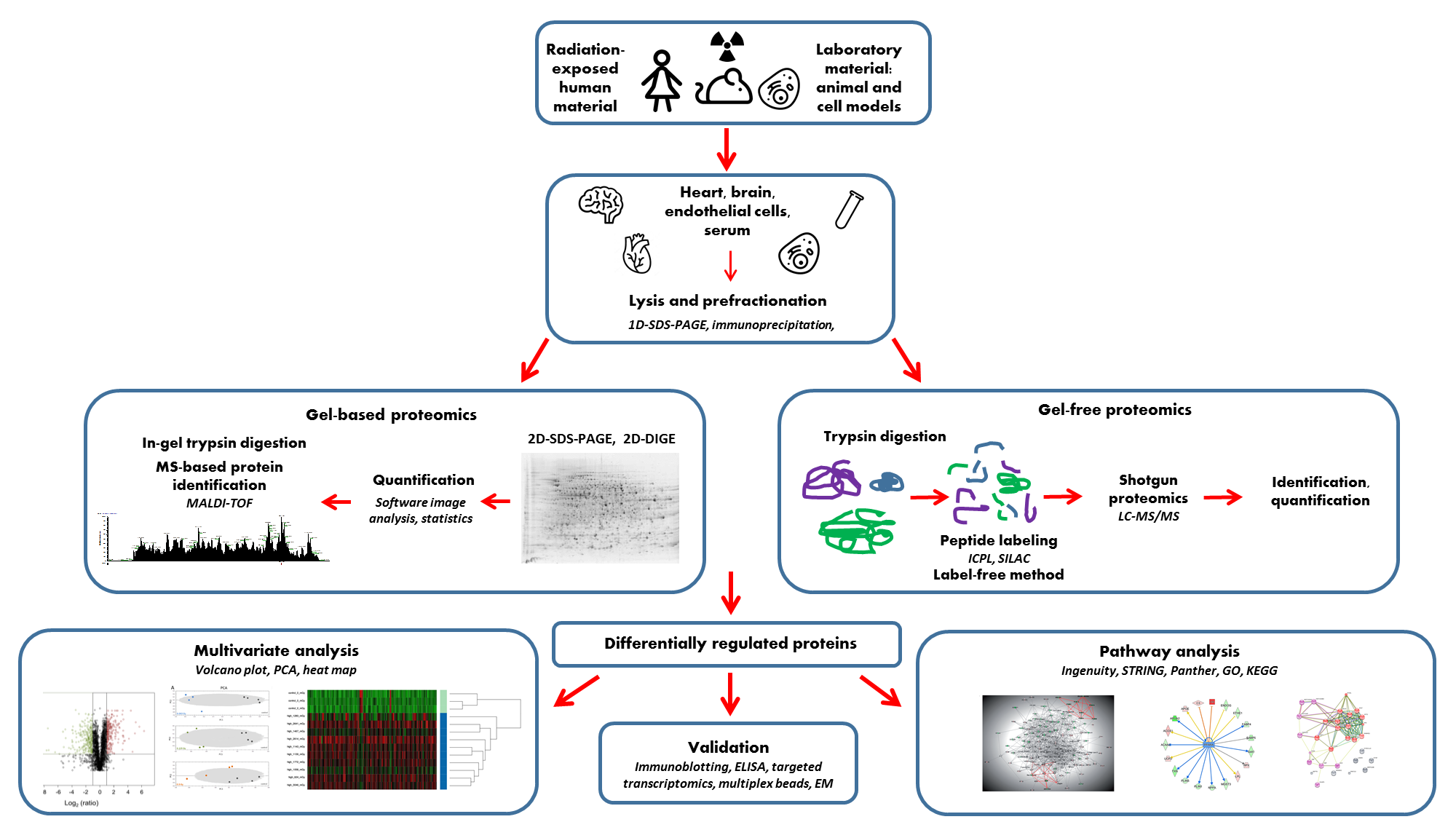
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**Figure 1.** Proteomics workflow describing methods used in this article.