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Release of redox enzymes and micro-RNAs in extracellular vesicles, during infection and inflammation

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Many studies reported that redox enzymes, particularly thioredoxin and peroxiredoxin, can be released by cells and act as soluble mediators in immunity. Recently, it became clear that peroxiredoxins can be secreted via the exosome-release route, yet it remains unclear how this exactly happens and why. This review will first introduce briefly the possible redox states of protein cysteines and the role of redox enzymes in their regulation. We will then discuss the studies on the extracellular forms of some of these enzymes, their association with exosomes/extracellular vesicles and with exosome micro-RNAs (miRNAs)/mRNAs involved in oxidative processes, relevant in infection and inflammation.

Compartmentalization of protein thiols/disulphides

The redox state of the cytoplasm, mainly due to the high reduced/oxidized glutathione (GSH/GSSG) ratio, is highly reduced while the extracellular environment is highly oxidized [1, 2]. For this reason, cytoplasmic proteins have very few structural disulphides while secretory proteins (e.g., albumins) have few reduced cysteines [3-5]. In an oversimplified view, the redox state of protein thiols is just determined by the chemical equilibrium with the GSH/GSSG couple and the fact that the endoplasmic reticulum has a low GSH/GSSG ratio is important for the formation of structural disulphides [1].

In recent years, the focus on reversible forms of oxidation of the thiols in protein cysteines has shown a more complex picture. In fact, many cysteines considered “free” according to the protein databases are only free from involvement in structural disulphides, but often oxidized to form sulfinic/sulfenic acids or mixed disulphides with glutathione or cysteine (often defined as glutathionylated or cysteinylated proteins, respectively) [6], suggesting that the compartmentalization of the redox state of protein

cysteines should be reviewed accordingly [7]. The proteins associated with the plasma membrane are at the interface with the cytoplasm and the extracellular environment and, despite the oxidizing environment on the outside, are rich in reduced cysteines that can be detected as surface thiols [8, 9].

Protein thiol/disulphide oxidoreductases

The redox state of protein thiol/disulphides is regulated not only by the GSH/GSSG ratio but also by a number of enzymes collectively defined as protein thiol/disulphide oxidoreductases (PTDORs), including thioredoxin (TXN), peroxiredoxin (PRDX), glutaredoxin (GRX) and protein disulphide isomerase (PDI) [10-12][1]. These enzymes catalyse the oxidoreduction of protein thiols or disulphides in a wide range of thiol-disulphide exchange reactions of different specificity. To these we should add sulfiredoxin (SRXN), which reduces the sulfenic acid of PRDX (that results in an inactive enzyme) to the free thiol [13], and quiescin oxidase (QSOX), a sulfhydryl oxidase that forms a disulphide from a thiol by direct oxidation [14].

In general, it is thought that TXN, GRX and SRXN are acting as reducing enzymes involved, mainly, in the reduction of protein disulphides (TXN), mixed disulphides (GRX) and sulfinic acid (SRXN). In contrast, PDI is mainly an oxidant enzyme, catalysing the formation of structural disulphides in the endoplasmic reticulum [1].

Many studies refer to PTDORs (except PDI) as “antioxidant enzymes”; although PRDXs are the only enzymes that can detoxify hydrogen peroxide (H_2O_2), these use TXN as the electron donor for their peroxidase activity and thus TXNs participate in the antioxidant action of PRDXs. Because some PRDXs (the so-called 2-Cys PRDXs) can be inactivated by overoxidation of active site cysteine during the catalytic reaction [15], and this is reversed by SRXN, we may consider also SRXN as supporting the peroxidase, antioxidant, activity of PRDX [13].

We have depicted the main biochemical activities of the main enzymes that will be mentioned in this review in Figure 1.

However, PTDORs have key biochemical functions independent of their “antioxidant” (i.e., scavenging reactive oxygen species, ROS) actions but related to their reducing activity. In particular, TXN was originally described as the cofactor for ribonucleotide reductase [16] and, in certain circumstances, GRX

[1] We have adopted here the use of the singular (TXN, PRDX) but for many of these enzymes several forms are present (e.g. PRDX1 to PRDX6).

can do the same [17]. Yet, both these enzymes have broad substrate specificity and can reduce a large number of proteins, with GRX preferentially reducing glutathionylated proteins [18]. Finally, when attempting to classify enzymes as reducing or oxidizing, one has to consider that some enzymes can catalyse both oxidations and reductions, depending on the environment. Significantly, TXN can catalyse the oxidation of protein thiols to disulphide bonds, like PDI, in an oxidizing environment [19]. It should also be noted that some PTDORs require other enzymes and substrates to catalyse some reactions. For instance, TXN can reduce protein disulphides but requires TXN reductase (which, in turn, requires NADPH), while PRDX requires TXN and TXN reductase for the reduction of H_2O_2, and yet these cofactors may not be present extracellularly.

Earlier studies on extracellular TXN and PRDX and their roles in immunity

While TXN and PRDX are usually studied for their intracellular activities, they have also been described as released by the cells in culture and present in circulation in plasma (reviewed in [20]). Extracellular TXN was originally described in the laboratory of Junji Yodoi as a cytokine released by leukaemia cells (Adult T-cell leukaemia-derived factor, ADF) that upregulates IL-2 receptors on T cells [21]. Upon sequencing, the protein turned out to be TXN [22]. Other studies have shown that extracellular TXN has multiple immune-related activities, enhancing cytotoxicity of eosinophils [23] and promoting leukocyte migration [24].

Various PRDXs were also described in plasma in multiple disease conditions, and studies showed that PRDX can enhance NK cell activity and has antiviral properties [25, 26].

Mechanism of secretion: exosome-targeting following protein oxidation

In vitro studies on TXN secretion by tumor cells clarified that this protein does not have a signal sequence for targeting through the Golgi and therefore does not follow the classical secretory pathway[2] [27, 28]. Likewise, of the secreted PRDXs, only PRDX4 has a signal sequence indicating that the others,

[2] Some argued that the term “secretion” is not appropriate if a protein is not released through the classical pathway. However, in this review we use this term in a broader meaning where a protein is not merely released as a result of cell death.

and particularly the 2-Cys PRDXs (PRDX1 and PRDX2), must also be secreted by a non-classical mechanism.

In 2002, we found that, in human monocytes, TXN1 can undergo glutathionylation of a non-active site cysteine, and suggested a role for this post-translational modification in its secretion [29]. Later, in redox proteomics studies, we identified PRDX1 and PRDX2 as proteins undergoing glutathionylation in a mouse macrophage cell line [30-33]. Peroxiredoxins 1 and 2 released by macrophages in response to the inflammatory stimulus, lipopolysaccharide (LPS) are present in the exosome fraction of the releasate and mutagenesis studies have shown that exosome targeting requires the formation of PRDX1/2 homodimers [34], linked by one or two disulphide bridges. However, secreted PRDX1 and PRDX2 are also glutathionylated, suggesting that two different types of cysteine oxidation are required, an intermolecular disulphide and a mixed disulphide with GSH [31], as depicted in Figure 2. To our knowledge, however, there has been no attempt to investigate whether glutathionylation is also important for PRDX1/2 secretion.

Likewise, TXN1 released by a human macrophage cell line stimulated with phorbol ester is associated to exosomes [35]. This suggests that the exosome route might be used by other redox enzymes.

We therefore searched a curated database of extracellular vesicles-associated proteins (Vesiclepedia, http://microvesicles.org/)[36]. According to this, the enzymes discussed above are vesicle-associated (Table 1). I Interestingly, also the mRNAs encoding for these same enzymes have been reported in vesicles.

Activities of redox enzymes and exosomes in infection and inflammation

This section will discuss the literature on redox enzymes and exosomes in the context of infection and inflammation. Beyond the purpose of this review, an abundant literature exists on the role of redox enzymes in B- and T-cell immunity (e.g., the ADF activity of TXN) and cancer that will not be discussed here.

With respect to TXN and PRDX, two major lines of evidence exist in the literature. The first supports the hypothesis that these enzymes act as antioxidants, and potentially anti-inflammatory mediators [37-39]. The second reinforces the hypothesis that TXN/PRDX can act as pro-inflammatory signals or cytokine-like molecules [11, 24, 40].

If the enzymatic, mostly reducing, activity of PRDX and TXN has been linked to their anti-inflammatory role, other studies pointed at an opposite effect as stimulators of innate immunity and inflammation, not necessarily connected to their enzymatic function. In recent years, several studies described potentially pro-inflammatory activities of TXN and PRDX. We wrote above that TXN was found identical to the previously characterized ADF, a T-cell growth factor. A truncated form of TXN induces IL-12 secretion in monocytes and promotes a Th1-type response [41, 42], while both full-length and truncated TXN have chemotactic activity towards different types of leukocytes [24, 42]. More recently, PRDX1 and 2 were shown to induce the production of inflammatory cytokines in macrophages, possibly via the activation of TLR4 [30, 33, 43]. Secretion of PRDX1 and 2 was induced by a variety of proinflammatory stimuli suggesting that they may amplify the inflammatory response [30, 43]. Inhibition by specific antibodies against PRDXs (not only PRDX1 or 2) decreases neuroinflammation and expansion of injury following cerebral ischemia in mice [33].

Although the indirect evidences could suggest that PRDX might directly interact with TLR4 [43], pointing at a redox-independent mechanism for its proinflammatory activities, this is still a hypothesis and other mechanisms are possible. We have suggested that, extracellularly, PRDX could act as a thiol oxidant because its peroxidatic action would require the presence not only of TXN, but also TXN reductase and a NADPH-generating system [30]. Extracellular vesicles from inflammatory conditions can produce ROS via NADPH oxidase (NOX) [44, 45] and thus could participate in the extracellular oxidation of PRDX and TXN. The release of PRDX1 and 2 in inflammatory conditions can have other consequences, beyond the promotion of inflammation as danger signals. In fact, we reported that LPS induces the release of preformed PRDX and results in a significant loss of the intracellular form of this enzyme [30]. Interestingly, combined treatment with IFN-gamma and LPS further reduces PRDX1 intracellular levels due to the activation of proteasomal degradation [46]. The loss of a peroxide-detoxifying enzymes due to the combination of increased degradation and vesicular release could make the intracellular environment more oxidizing (Figure 3), perphaps facilitating the killing of intracellular pathogens [46].

Overall, the proinflammatory activities of PRDX1 and 2 reported *in vitro* [30, 43] and *in vivo* [33, 47] enlists them among the damage-associated molecular patterns (DAMPs) [48]. These include a vast array of proteins, nucleic acids and small metabolites that can trigger inflammation in response to cell damage, explaining how inflammation develops in sterile conditions (also known as sterile inflammation), for example inischemia, during autoimmunity or after trauma (e.g. burns, etc.) [48]. Relevantly, another protein known as a key DAMP, high-mobility group box 1 (HMGB1) [49] has been

reported to undergo redox modification in specific cysteine residues affecting its immune function [50]. Similarly, HMGB1 is also secreted via a vesicle-mediated pathway [51]. However, while specific cysteine mutagenesis blocks the secretion of PRDX1 and 2, we found that, HMGB1 secretion does not rely on the presence of specific cysteines, at least in TNF-treated HEK293 cells [34].

Oxidative stress and micro-RNA pathways relevant in inflammatory and infection conditions

Intracellular levels of PRDX and other antioxidant proteins are regulated by oxidative stress response, increasingly reliant on the involvement of micro-RNAs (miRNAs, [52]), that collectively comprise about ~2,656 human species [53]. Interestingly, similar to PRDX, there seems to be a dual interpretation of the possible roles of miRNAs in biology. At the intracellular level (either in their original source cells or in target cells that have received them as a result of intercellular exchange), miRNAs are involved in post-transcriptional regulation of gene expression by interacting with target mRNAs, in the presence of the RNA-induced silencing complex (RISC), including the RNA silencing endonuclease, Argonaute 2 (Ago2) [52]. About 60% of genes involved in human biology and disease can be targeted by miRNAs [54, 55]; multiple transcripts share homology target sequences complementary to the miRNA seed regions, hence a single miRNA affects the expression of multiple genes, as part of a wide network. In this context, miRNAs that directly bind to target inflammatory cytokine genes may mediate anti-inflammatory properties. Nonetheless, cellular miRNAs may well bind other target mRNAs regulating complex networks driving or inhibiting inflammatory cytokine expression [56].

Conversely, when secreted, miRNAs may act as DAMPs which would bind to TLRs (e.g. TLR7, potentially targeted by vesicular miRNA [57, 58] and could therefore play a proinflammatory role. In thissection, we focus on miRNA involvement in oxidative stress responses and impact on PRDX, with an emphasis on potential extracellular exchange mechanisms of relevance in infection and immunity.

Cellular content of miRNAs can be modulated by ROS and *vice versa* (Figure 4). Environmental exposure to xenobiotics (e.g., benzene and other aromatic hydrocarbon compounds), heavy metals (e.g., arsenic, cadmium) and even air pollution, noise and mental stress can upregulate ROS (and consequent inflammation), activating specific miRNAs pathways, some of which may be involved in xenobiotic toxicity, cell survival/function and disease (reviewed in [59]). Exposure to genotoxic agents (etoposide and radiation) activates miRNA pathways similar to those affected by oxidative stress (including miR-27a, miR125a, miR-29a and others) in human fibroblasts, pointing at a potential common miRNA

signature of cellular stress [60]. Further, infectious agents, allergens and inflammatory mediators (e.g., TNFα), as well as physiological ageing can additionally directly impact on ROS and miRNAs involved in oxidative stress responses (read further below for details). Oxidative stress, when leading to damage to cellular proteins and structures can regulate, in parallel, expression of miRNAs involved in autophagy and protein ubiquitination/degradation [61].

Enzymes that critically drive antioxidative responses against cellular stress induced by ROS can become direct targets of miRNAs, suggesting that miRNA regulation may modulate reparative pathways after ROS exposure. For example in endothelial cell lines, after exposure to H_2O_2, expression of TXN reductase (TXNR) (part of the TXN antioxidative system [62]) is upregulated thanks to the downregulation of miR-125a, which would otherwise bind to the 3' UTR of TXNR mRNA and suppress its translation [63]. The thioredoxin interacting protein (TXNIP) is a critical inhibitor of TXN and plays a critical role in the activation of inflammatory pathways, supporting oxidative stress damage [62], also relevant in diabetes. Imterestingly, a miRNA modulated by oxidative stress, miR-128 has been recently shown to target TXNIP ([61, 64], potentially suggesting an indirect pathway through which miRNAs can support antioxidative responses. In a fish model of oxidative stress driven by ageing, miR-200 family members are associated with ROS in ageing hearts and miR-29 plays a protective role against fibrosis/hypoxia, by affecting a series of target genes encoding for collagen and DNA methyltransferases in cardiac cells [65]. Downmodulation of miR-29 levels in the heart tissue increased levels of multiple hypoxic marker mRNAs (Hk2, Epoa, Hmox1a and Ldha), associated with hypertrophic/fibrotic responses. Human cardiac fibroblasts also upregulated miR-29 family members after exposure to H_2O_2, potentially impacting on the expression of direct targets (DNMT3a/3b and col1a1, col3a1, col11 and col15), involved in fibrotic damage. Conversely, hypoxia decreased miR-29 expression, upregulating fibrotic damage targets [65].

Some of the ROS-modulated miRNAs target transcription factors, such as Nrf2 [66] critical to regulate antioxidative responses, through binding to the antioxidant responsive element (ARE) in the promoter of target regulated genes. For instance, in a rat model of chronic heart failure (CHF), typically accompanied by oxidative stress, it was shown that Nrf2 protein levels are downregulated in the affected heart ventricles, leading to a reduction of downstream antioxidant enzymes, including superoxide dismutase (SOD), catalase and heme oxygenase-1 [67] . However, Nrf2 mRNA was not found to be downregulated in the same samples, pointing at a potential translational control mechanism mediated by miRNAs. Consistent with this hypothesis, miR-27a, miR-28a-3p and miR-34a levels were found to increase in the myocardium of CHF rats, as well as these miRNA mimics inhibited the induction of Nrf2 expression, in

TNFα stimulated cardiomyocytes [67]s. Similarly, in homozygous sickle cell disease, miR-144 decreases Nrf2 expression in reticulocytes ultimately leading to decreased antioxidative pathways in erythrocytes, a potential mechanism behind reduced oxidative stress tolerance in those patients [68]. Further, miR-27a and miR-144 (as well we miR-153 and miR-142-5p) were reported to directly target Nrf2 protein, and thus downstream redox balances, in neuronal models (neuroblastoma cell lines, SH-SY5Y), relevant for neurodegenerative disease [69]s.

During ageing, oxidative stress increases in rat livers accompanied with loss of Nrf2 protein and a steady rise in the levels of miR-34a and miR-93, which were reported to dampen Nrf2 expression in transfection experiments [70]s. In liver inflammation associated with non-alcoholic fatty liver disease (NAFLD), an antioxidant/anti-inflammatory polyphenol, polydatin, targets miR-200a which in turns downregulates KEAP1, the negative regulator of Nrf2, favouring the release and nuclear translocation of active Nrf2 which then activates downstream antioxidative pathways [71]. The authors showed that fructose (a driver of NAFLD) downregulates miR-200a, thus inhibiting Nrf2 with a consequent exacerbation of liver inflammation [71]. Similarly, inhibition of miR-377 by allopurinol (an inhibitor of the ROS-generating enzyme xanthine oxidase) and the antioxidant pterostilbene, protects against oxidative damage in the glomeruli, and the authors suggested that miR-377 may target SOD1 and SOD2 expression [72].

In gastric cancer cells lines, it was shown that miR-24 can directly target expression of PRDX6 [73]. This is relevant in pre-cancerous gastric lesions associated with *H. Pylori* infection, which causes oxidative stress, reducing expression of miR-24 and increasing that of PRDX-6, as part of an antioxidant repair mechanism; however, this balance is lost in gastric cancer lesions [73].

In a model of cockroach allergy, related to asthma, the allergen increased oxidative stress and inflammation, promoting COX-2 upregulation, directly regulated by miR-155 (in fact, COX-2 mRNA 3' UTR contains three miR-155 target sites), promoting oxidative stress and lung inflammation. Interestingly in this case, miR-155 levels were promoted by the allergen exposure, and this miRNA increased the stability of its target mRNA; thus, inhibition of miR-155 might reduce ROS in asthma, by lowering COX-2 and decreasing inflammation [74]. Consistent with this hypothesis, severe rather than mild/moderate asthma patients have higher plasma levels of miR-155 [74]. Thus, miRNAs may participate in intracellular pathways in response to oxidative stress which may contribute to damage response mechanisms within the cell, and even mediate protection/repair mechanisms, for example, like seen in the above-mentioned case of miR-29, against age-related increase of ROS.

In addition to the regulation of intracellular miRNAs, there is evidence that certain miRNAs (as well as other RNA species[75]) can be present in blood as protein complexes and/or extracellular vesicles [76] secreted via the exosome route [77, 78] (Figure 4). These can be used as markers of various disease often leading to the detection of single miRNA biomarkers [79], although the function of such miRNAs remains broadly unclear.

Many studies have focused on the extracellular miRNAs in response to oxidative stress in a variety of conditions, but this is especially relevant in cardiovascular disease, liver, stroke and other brain disease [80]. These studies pointed at specific miRNAs being affected, such as miR-126-3p, miR-122-5p, miR-223-3p, miR-192-5p, miR-1224-5p among many others [80]. For instance, circulating miR-122 and miR-200a are associated with HIV and HCV coinfection, more likely to accumulate in vesicles of individuals at risk of developing fatal liver disease [81]. In addition, levels of these miRNAs positively correlated with those of inflammatory cytokine, IL-6 and other liver damage biomarkers [81]. A recent analysis of miRNAs in plasma-derived vesicles from HIV+ and HIV- individuals revealed a predominance of certain miRNA species in vesicles (miR-26a-5p, miR-21-5p and miR-148-3p among many others), including few differentially expressed in infection. Interestingly, the accumulation of plasma (vesicle) miRNAs (in particular: miR-21-5p, miR-27b-3p, miR-146a-5p, and miR-423-5p) positively correlated with oxidative stress biomarkers such as cystine, cysteine, cysteine-s-sulfate, and oxidized-cys-gly [82].

We have found that plasma miRNAs are extensively and globally perturbed in conditions of severe infection (sepsis) and inflammation during systemic inflammatory response syndrome (SIRS), in humans [83, 84]. Both these conditions are associated with oxidative stress that amplifies the inflammatory response and might aggravate the pathology [85-87]. We defined a subset of circulating miRNAs specifically regulated by SIRS and infection that we termed Circulating Inflammatory-Relevant miRNAs (CIR-miRNAs) [83]. The levels of CIR-miRNAs globally increased in sepsis and SIRS patients, relatively to nnon-sepsis/SIRS controls. However sepsis, compared with SIRS, was associated with relative lower amount of several CIR-miRNAs [83], in agreement with previous studies reporting decrease of individual miRNA biomarkers in sepsis compared to SIRS [88, 89].

These studies are consistent with the possibility that several miRNA species may be “sponged”, i.e., sequestered, by other non-coding RNAs [90] or mRNAs (the so-called “sponges”), potentially including actual miRNA-targets upregulated in sepsis [91]. Alternatively, these could be removed from (or exit) the blood circulation, in sepsis more than in other inflammatory conditions, globally affecting miRNA networks. Some of the CIR-miRNAs (in particular, miR-378a-3p, miR-30a-5p, miR-30d-5p, and miR-192-

5p) correlate with the severity of systemic inflammatory disease and the levels of circulating PRDX-1 in of SIRS patients [84]. CIR-miRNAs can be also secreted *in vitro* by immune cells upon stimulation with bacterial antigens [84]. In critically-ill patients (sepsis and SIRS), CIR-miRNAs inversely correlate with levels of inflammatory cytokines [83]. Yet, in these conditions, the mechanistic relationships between miRNAs, oxidative stress mediators and inflammatory cytokines remain elusive. The presence in the circulation of CIR-mRNAs and their correlation with PRDX1 levels during severe inflammatory pathology strengthen the hypothesis that these may be secreted by inflammatory cells, perhaps in exosomes/extracellular vesicles (EVs), possibly accounting for global/network changes detected in plasma miRNAs during severe inflammatory disease.

Exosome RNA exchange in oxidative stress.

Upon exposure to oxidative stress, various cell types are capable of producing exosomes (small vesicles ranging in size between 30/50-150 nm) and other (larger) EVs (100/150-1500 nm) [92, 93], beyond apoptotic bodies (100-5000 nm) [94]. Interestingly these can contain a series of proteins (including antioxidant proteins, such as Nrf2 and Prdx-1[93] as well as a range of RNA species (Figure 4). Exosome/EVs contain a relatively stable pool of proteins, including structural and chaperone proteins [95], while they vary widely in RNA content [92]. Importantly, it has been shown that exosome transfer of RNA species between origin and recipient/target cells can shape gene expression in the latter [94, 96].

It is still unclear how specific miRNAs and mRNAs end up being packaged into exosomes/microvesicles (Figure 4), but evidently the specific accumulation of certain RNA species in exosomes/EVs, and not in their source/producer cells, suggests that specific RNA-sorting mechanisms might exist, dependent on the availability of cellular targets [97]. In these respects, it should be noted that certain miRNAs can interact with mRNA encoding for antioxidant mediators. For example, miR-24-3p targets the 3'UTR of PRDX-6 mRNA [73], and this could be an important mechanism to control gastric tumour cell progression in the presence of chronic inflammatory stimuli induced by *H Pylori* infection.

Micro-RNAs may be co-sorted and packaged into exosomes/EVs, together with their target mRNAs (e.g., miR-24-3p and PRDX-6 mRNA), or possibly even miRNA sponges. This would point at an eventual subcellular co-localization of miRNAs with target mRNAs or sponges at the time of exosome packaging (Figure 4). For instance, P-bodies (different from stress granules) are known to be involved in miRNA/mRNA storage/degradation [98] and these structures tend to be generated in cells under

conditions of stress and/or in cancer [99]. Although not morphologically different from P-bodies [98, 99], GW-bodies are characterised to be richer in GW182 and Ago2 protein composition as well as non-translating RNAs [100]s and more specifically associate with the multivesicular body [101]. In this respect, it should be considered that the miRNA:mRNA interaction would rely on the presence of the RISC complex and recently Ago2 has been shown to play a role in miRNA sorting in exosomes [102]; yet so far only relatively few studies have reported the presence of Ago2 in protein analyses of EVs [103, 104]. Nevertheless, a large proportion of proteins found in vesicles are RNA-binding proteins that could also play a role in co-sorting miRNA/mRNA or, alternatively, it could well be that miRNAs do not interact directly with mRNA in exosome/EVs [94], as evidence suggests that (at least macrophage) cells tend to direct excessive miRNAs towards the exosome/EV pathway to regulate intracellular miRNA:mRNA balances [97] (Figure 4).

Whether P/GW-bodies are regulated by oxidative stress remains elusive. Intriguingly, GW/P- bodies have been associated with autoantigens, in autoimmunity [105]. In this respect, it was found that an autoantigen associated with autoimmune primary biliary cirrhosis (and other autoimmune conditions), Ge-1[106] is structurally involved in P-bodies that change subcellular localisation after exposure to arsenite-induced oxidative stress [107, 108]. Arsenite treatment in HeLa cells induces the accumulation of oxidized RNA species, particularly 8-hydryoxyguanine (8-oxoG) [109], associated with human disease [110, 111]. These oxidized RNA species localize in cytoplasmic foci of HeLa cells distinct from stress granules and P-bodies, named oxidized RNA bodies (ORBs, [109], Figure 4). ORBs are absent in mitochondria (thus, oxidized RNA species would not derive from these organelles), yet there is evidence of ORBs localization in the cell nucleus, additionally [109].

While there is evidence to suggest that localization of mRNAs in the cell can impact on balances between translation and degradation [112], it remains unclear whether oxidized RNA species are sorted in exosomes and/or impact on the miRNA:mRNA balance and intercellular communication. In Caenorhabditis elegans, knockdown of EDC-3, an enzyme involved in mRNA decapping (and hence stability) that promotes mRNA degradation in P-bodies leads to increased worm longevity and resilience to ageing, mediated by the Nrf-1/2 homolog, SKN-1, critically involved in antioxidative pathways [113]. Thus, regulation of decapping enzymes/mRNA degradation in P-bodies may be counterbalanced with Nrf2/SKN-1 reparative pathways, presumably due to the potential coupling of mRNA degradation with oxidative stress. Consistently, the enhanced survival of the EDC-3 deficient worms is abolished by knockdown of SKN-1, associated with an increase number of cytoplasmic P-bodies, all of which is

partially rescued by treatment with ROS scavenger, N-acetylcysteine [113]. In conclusion, more research is needed to clarify the relationships between oxidative stress, RNA degradation/oxidation and RNA species intracellular localization as well as sorting/exchange in exosomes, especially in humans (Figure 4).

Role of exosome/EV molecules in transferring protection against oxidative stress

There are several studies on the potential anti-inflammatory action of TXN and PRDX associated with their "antioxidant" action (for instance: [114-116]). Although these studies deal with redox regulation by intracellular enzymes, that are not the subject of this review, extracellular TXN or PRDX can potentially be taken up by other cells to increase their intracellular levels (Figure 4). Transfer of PRDX1 into macrophage cells decreases the production of LPS-induced inflammatory cytokines [117]. Administration of recombinant TXN1 has anti-inflammatory activity *in vivo* [118, 119]. It is conceivable that EVs could transfer these enzymes to target cells. In fact, exosome-mediated transfer of catalase and superoxide dismutase has been shown to occur from glial cells to neurons at biologically-relevant levels [120].

Likewise, it has been proposed that exosomes generated in conditions of oxidative stress may transfer tolerance to oxidative processes to recipient cells via miRNAs. Mast cell-lines, a particular type of immune cells involved in allergy and tissue repair mechanisms (e.g. during wound healing) respond to ROS by secreting exosomes that in turns confer resistance to cell-death induced upon exposure to H_2O_2 , in recipient target cells [92]. Such exosome-driven tolerance to oxidative stress was reversed by UV exposure which ablates RNA species in exosomes [92]. Furthermore, mRNAs in exosomes and their correspondent source/producer cells were analysed before and after exposure to ROS. In exosomes-producing cells, differential gene expression (DGE) was found to be much reduced compared to differential content of mRNAs in exosomes, suggesting that active/specific sorting of mRNA species into exosomes is substantially impacted by ROS/oxidative stress. Interestingly, the degree of oxidation, in terms of carbonylation of exosome/EV proteins was lower than that of the intracellular proteins [92], which might be compatible with the presence of antioxidant proteins, such as PRDX and TXN, in exosomes/EVs.

Exposure to ROS induces the production of exosomes containing functional mRNA encoding for Nrf2 and antioxidants enzymes that could be exchanged, eventually transferring antioxidant defence mechanisms

in target epithelial-like/somatic cells [93]. After exposure to H_2O_2, retinal epithelial cells increased secretion of small EVs, potentially promoting angiogenesis by decreasing the content of miR-302a and miR-122, among many other miRNAs. Also, in this case the miRNA content after ROS exposure differed significantly in the source cells compared to EVs [121].

In the future, exosome-derived PRDX (and other antioxidant enzymes) may become instrumental to mediate antioxidative properties beneficial in therapeutic settings. For instance, it was recently found that human induced pluripotent stem cells (iPSCs) can secrete exosome/EVs capable of reversing the damage of oxidative stress/ROS upon intake in target senescent, mesenchymal stem cells (MSCs) [122]. Senescent MSCs showed decreased levels of intracellular PDRX1/2 (while accumulating ROS), loss of proliferative potential, and ageing, all of which was reversed by iPSC exosome/EVs. Interestingly, the iPSC exosome/EVs were rich in PRDX1/2 (more than PRDX4, 5 and 6) [122]. We previously found that plasma PRDX1 correlates with CIR-miRNA levels in SIRS, more than sepsis, patients [84]. Intriguingly, SIRS was also associated with a substantial increase of plasma CIR-miRNAs (higher than that seen in sepsis) [83]; this may suggest that PRDX1 plasma levels may be more stable in the plasma of SIRS, rather than sepsis patients, potentially indicative of antioxidative/inflammatory pathways potentially activated in SIRS (note: SIRS is much less lethal than sepsis).

It should be noted that there are also cases when the exchange of exosome/EV miRNAs promotes oxidative stress, apoptosis and tissue damage. Upon inflammatory stimulation (TNFα), rat cardiac fibroblasts release exosomes/EVs containing miRNAs (including regulators of Nrf2, miR-27a, miR-28a and miR-34a) that can be exchanged with cardiomyocytes, leading to a decrease of Nrf2 expression in target cells, detrimental for antioxidative defence mechanisms [67], as well as supporting hypertrophic responses. In this context, systemic administration of an inhibitor of miR-27a helped improve heart contractility [67] . In another example, it has been shown that in Type-2 diabetes (T2D) patients have increased levels of miR-15a in blood. Under T2D (high glucose) treatment, pancreatic β-cells were shown to secrete miR-15a in CD63+/CD81+ exosomes. Upon uptake in retinal (Müller) cells, transferred pancreatic miR-15a increased ROS generation in recipient cells, leading to apoptosis, via Akt3. Retinal intake of pancreatic exosome/miR-15a was further validated *in vivo* in mice with hyperglycaemia after high-fat diet [123].

In conclusion, infection, inflammation/irritants, allergens and ageing lead to oxidative stress, increasing ROS that drive wide changes and oxidative damage to cellular structures and molecules, well beyond proteins and lipids, and encompassing miRNA/mRNA species (Figure 4). These can start negative

feedback loops that lead to protective antioxidative responses (mediated by Nrf2, PTDORs, etc.). Still, there are cases of ROS-induced miRNAs that can promote inflammation and oxidation of RNA species further driveing inflammation, that can also be induced by PRDXs when these are acting as DAMPs. More research is needed to understand how localisation of miRNA/mRNA species in specific regions of the cytosol (P/GW-bodies, ORBs etc.) impacts on packaging of miRNA, mRNA and proteins into exosomes, especially since the mechanisms of sorting miRNA/mRNA species (and proteins, including PRDX and Nrf2) in exosomes remain largely undefined.

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Table 1. Presence of redox enzymes and their mRNAs in microvesicles.

Enzyme	Protein	mRNA
GLRX1, 2,3,5	x	x (GLRX1 and 3)
PRDX1-6	x	x
TXN1-2	x	x
TXNRD2	x	x
SRXN1	x	x
QSOX1-2	x	x

Reference: from http://microvesicles.org, updated 30/12/2020

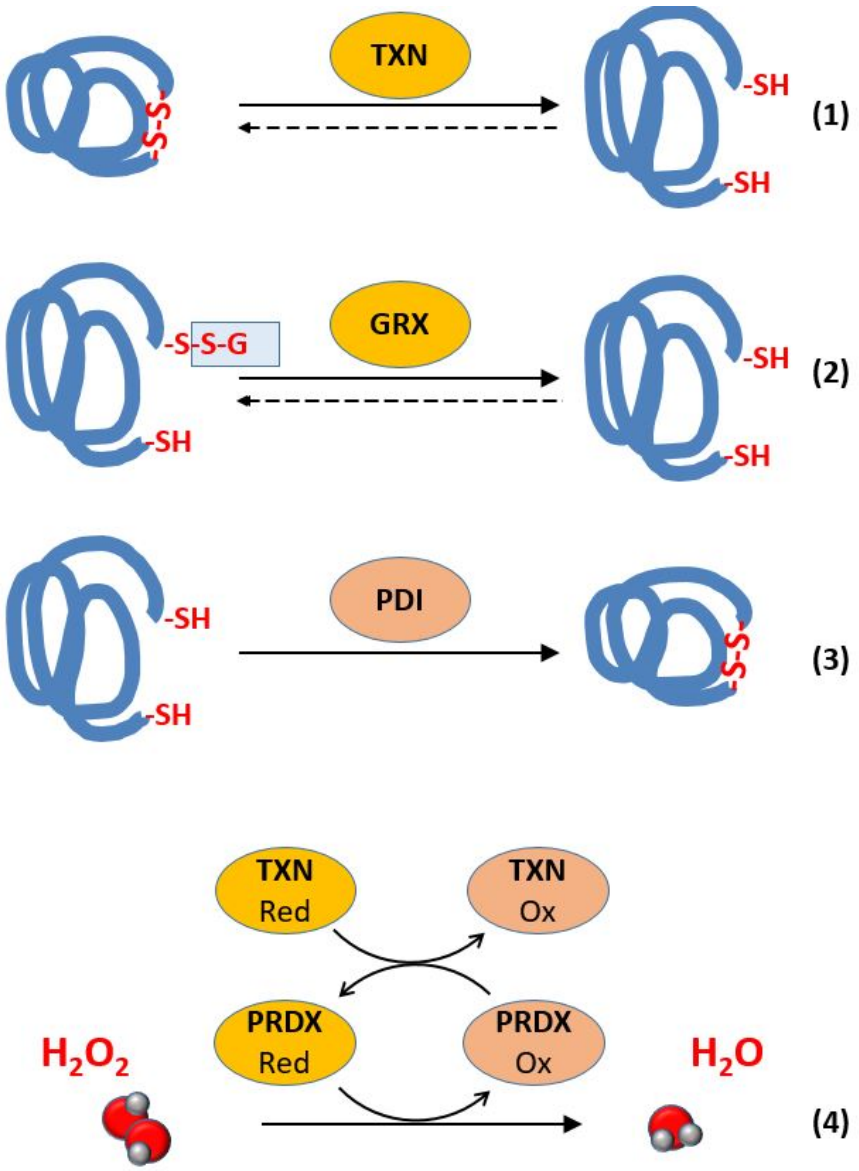

Figure 1. The main biochemical activities of TXN, GRX, PDI and PRDX. TXN and GRX catalyses the reduction of protein disulphides (1) and (2), with GRX being specific for glutathionylated proteins. Both enzymes catalyse a thiol-disulphide exchange, but work mainly as reductant. PDI acts mainly as an oxidant and catalyses the formation of (mostly) structural disulphides during protein folding (rarely reversible) (3). PRDX is a peroxidase that reduces hydrogen peroxide to water using reduced TXN as the electron donor (4). Both reactions (1) and (2) lead to the formation of oxidized TXN. This is then reduced back to reduced TXN by the NADPH/TXN reductase system (not depicted here).

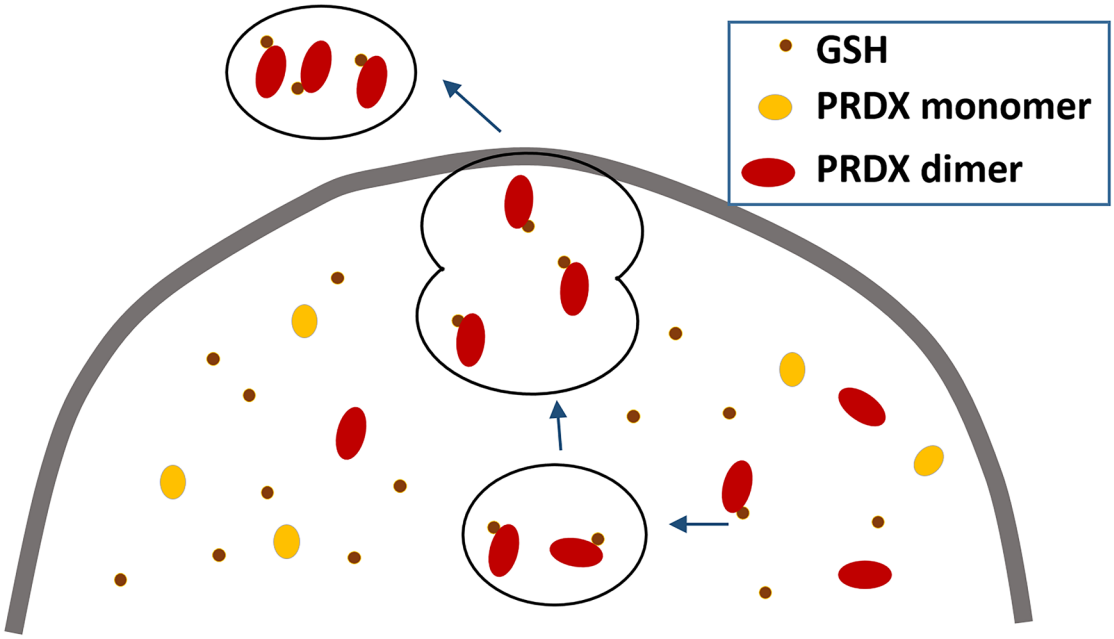

Figure 2. Intracellular PRDX1/2 forms glutathionylated, disulfide-linked homodimers that are required for the protein to be released via the exosomal route in LPS-stimulated macrophages.

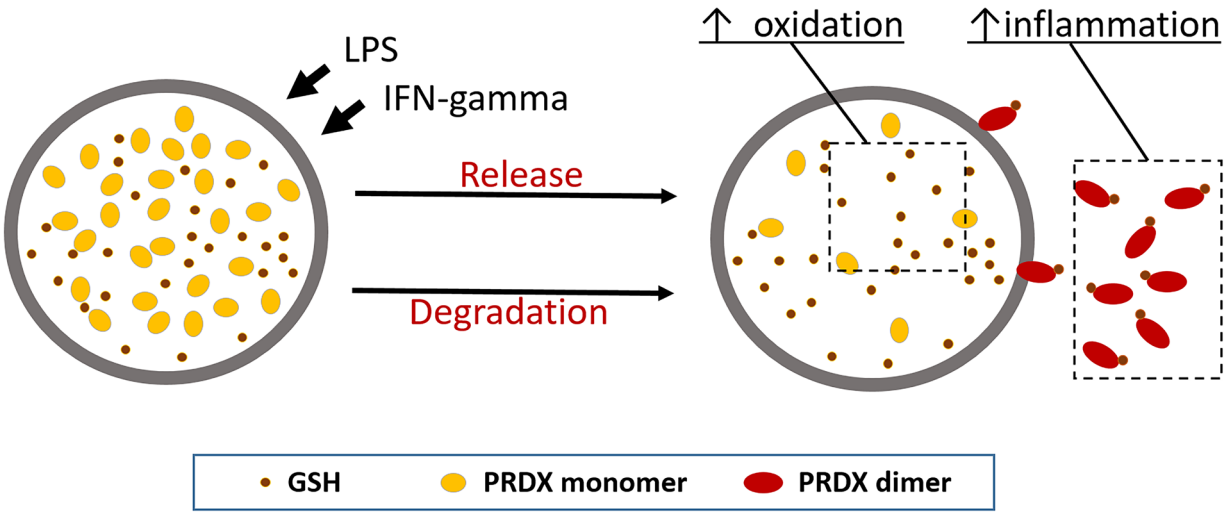

Figure 3. Possible role of PRDX release in inflammation. LPS induces exosomal release of PRDX1/2 and, in association with IFN-gamma, its proteasomal degradation. As a net result, macrophages PRDX levels are decreased resulting in a more oxidized environment. The PRDX released can exacerbate inflammation by triggering inflammatory cytokine production by TLR4 activation.

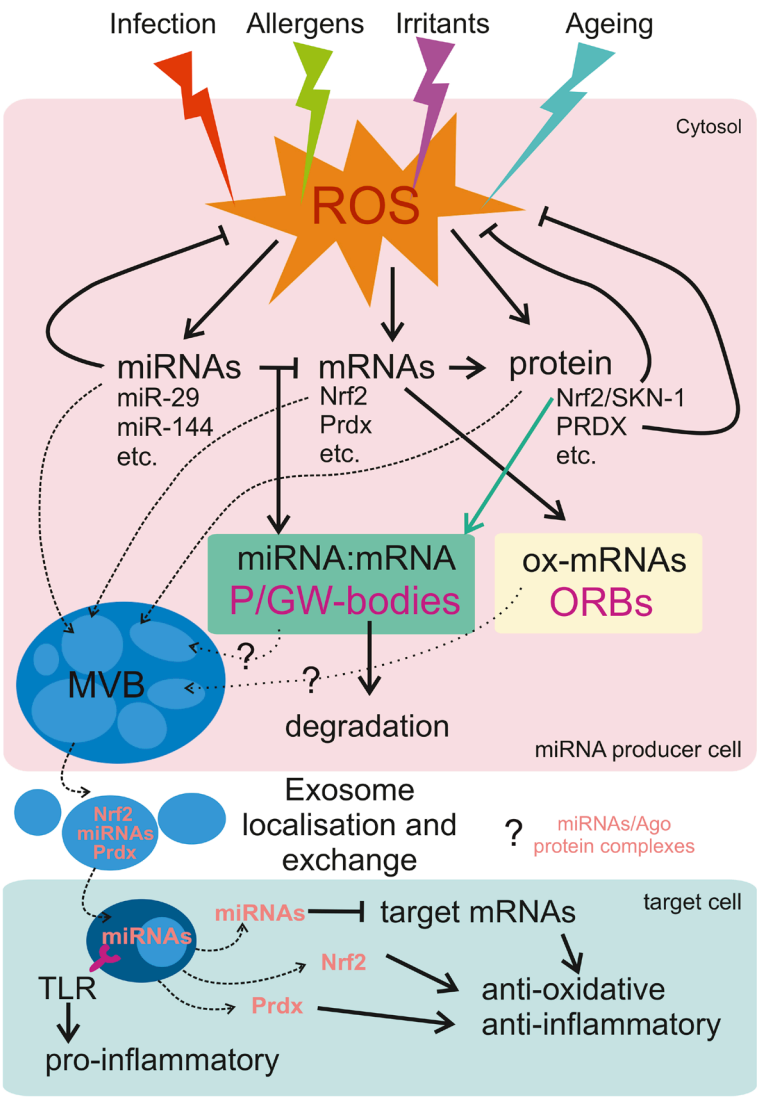

Figure 4. Interplay between ROS and miRNA/RNA networks intracellularly and in exosome/miRNA exchange. Infection (e.g., *H. Pylori*), inflammation/irritants (e.g., arsenite), allergens (e.g., cockroach allergens) and ageing lead to oxidative stress, increasing intracellular levels of reactive oxygen species (ROS) that can drive changes in miRNA levels and oxidation of RNA species, beyond impacting on gene expression/mRNA levels. In turns, miRNAs downregulate levels of target mRNA, after annealing to complementary target sequences, often located in the 3'UTR of the target mRNA (miRNA:mRNA). This interaction can interrupt and/or inhibit protein translation, potentially leading to decreased expression of inhibitors of Nrf2 (Keap1) or Nfr2 directly, respectively in anti-inflammatory and pro-inflammatory pathways. This can also drive the subcellular localization of miRNA:mRNA complexes in P/GW-bodies (green insert), specific cytosolic structures rich in specific protein composition, such as GW182/Ago2, decapping enzymes and other proteins involved in RNA

degradation/storage. ROS can impact on localization and number of P/GW-bodies, potentially via a Nrf2/SKN-1 pathway (green arrow). Oxidised RNA species can additionally localise to specific cytosolic structures, known as oxidised RNA bodies (ORBs, yellow insert); ORBs have been described as separate from P/GW-bodies. MiRNAs, mRNAs and proteins can be packaged into exosomes (light blue, and dotted lines), small vesicles originated from the multivesicular body (MVB, blue). Oxidative stress can impact on the amount and species of miRNAs/mRNAs and proteins localising in exosomes. However, sorting mechanisms and whether miRNAs/mRNAs traffic from P/GW-bodies and ORBs to exosomes remain unknown, notwithstanding the fact that mRNA cytosolic levels may impact on the sorting of miRNAs in exosomes. Oxidative stress/inflammatory disease drives the release of exosome/miRNAs and/or extracellular miRNAs (e.g., circulating inflammatory relevant miRNAs, CIR-miRNAs relevant in sepsis and systemic inflammation) correlating with the presence of antioxidative mediators, PRDX and even Nrf2 (or their mRNAs). Exosome/miRNAs intake in target cells (teal insert), via the endocytic pathway (dark blue) can activate TLR7 pro-inflammatory pathways, while release of miRNAs/PRDX/Nrf2 in the cytosol of the target cells may drive anti-inflammatory functions. Thus, miRNAs/PRDX intercellular exchange may have a dual nature: these can act as pro-inflammatory DAMPs, when they act as ligands of TLR7/4, or anti-inflammatory/oxidative mediators, when they interact with target mRNA/oxidised substrates.