Redox-related biomarkers in human cardiovascular disease - classical footprints and beyond

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ABSTRACT

Global epidemiological studies show that chronic non-communicable diseases such as atherosclerosis and metabolic disorders represent the leading cause of premature mortality and morbidity. Cardiovascular disease such as ischemic heart disease is a major contributor to the global burden of disease and the socioeconomic health costs. Clinical and epidemiological data show an association of typical oxidative stress markers such as lipid peroxidation products, 3-nitrotyrosine or oxidized DNA/RNA bases with all major cardiovascular diseases. This supports the concept that the formation of reactive oxygen and nitrogen species by various sources (NADPH oxidases, xanthine oxidase and mitochondrial respiratory chain) represents a hallmark of the leading cardiovascular comorbidities such as hyperlipidemia, hypertension and diabetes. These reactive oxygen and nitrogen species can lead to oxidative damage but also adverse redox signaling at the level of kinases, calcium handling, inflammation, epigenetic control, circadian clock and proteasomal system. The in vivo footprints of these adverse processes (redox biomarkers) are discussed in the present review with focus on their clinical relevance, whereas the details of their mechanisms of formation and technical aspects of their detection are only briefly mentioned. The major categories of redox biomarkers are summarized and explained on the basis of suitable examples. Also the potential prognostic value of redox biomarkers is critically discussed to understand what kind of information they can provide but also what they cannot achieve.

1. Introduction

Within the last decades the global burden of disease experienced a shift from maternal, perinatal and nutritional causes as well as communicable (infectious) diseases to non-communicable diseases (e.g. atherosclerosis, hypertension and diabetes), which may be best explained by the demographic shift in population, exacerbation of environmental pollution but also improved global nutrition, medication and sanitation [1,2]. The future projections of the World Health Organization (WHO), based on data of the Global Health Observatory, predict a 77% share of non-communicable diseases in the global burden of disease in 2030 [3,4]. In 2016, 44% of all global deaths caused by chronic non-communicable diseases were of cardiovascular origin [5], with ischemic heart disease (IHD) as a leading risk factor and a global prevalence of 128 million people as well as 9 million attributable premature deaths mainly caused by myocardial infarction (MI) and heart failure [6]. Importantly, non-communicable diseases represent or generate comorbidities (e.g. hyperlipidemia, hypertension or diabetes) that have great impact on cardiovascular risk and mortality [7]. These lines of evidence highlight the importance of comorbidities for the assessment of overall cardiovascular risk and the need for more and better biomarkers to characterize and provide early diagnosis of cardiovascular risk factors.

The mentioned comorbidities are all associated with an increased burden of oxidative stress [Fig. 1] [8,9], which is characterized by

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Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| 3-NT | 3-nitrotyrosine; |
| 4-HNE | 4-hydroxynonenal |
| 8-iso-PGFA | 8-iso-prostaglandin F2α |
| 8-OHdG | 8-hydroxy-2-deoxyguanosine; |
| 8-OHG | 8-hydroxyguanosine (also 8-oxoGlu) |
| AF | atrial fibrillation |
| AGE | advanced glycation endproduct |
| CAD | coronary artery disease |
| cGMP | cyclic guanosine monophosphate |
| CI | confidence interval |
| COPD | chronic obstructive pulmonary disease |
| CVD | cardiovascular disease |
| GSH | reduced glutathione |
| GSSG | oxidized glutathione disulfide; |
| HR | hazard ratio |
| HIF-1α | hypoxia-inducible factor-1α |
| IHD | ischemic heart disease |
| I/R | ischemia/reperfusion |
| LDL | low-density lipoprotein |
| MDA | malondialdehyde |
| MI | myocardial infarction |
| MPO | myeloperoxidase |
| NOS | nitric oxide synthase |
| NOX | NADPH oxidase |
| OR | odds ratio |
| RNS | reactive nitrogen species |
| ROS | reactive oxygen species |
| RR | relative risk |
| SMD | standardized mean difference |
| sNOX2-dp | soluble nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) 2-derived peptide; |
| SOD | superoxide dismutase |
| WHO | World Health Organization |
| XO | xanthine oxidase |

Fig. 1. Concept of redox biomarkers for comorbidities in myocardial infarction (MI). Identification of specific redox biomarkers for different comorbidities (e.g. hypertension, hyperlipidemia or diabetes) in major cardiovascular events (e.g. MI) would significantly advance redox diagnostic approaches. Currently most redox biomarkers belong to the category of „additive accumulation” that could at least be used as prognostic tools to identify patients with multiple comorbidities representing the group of highest cardiovascular risk. Heart cartoon from Servier Medical Art by Servier, licensed under a Creative Commons Attribution 3.0 Unported License.
excessive formation or insufficient break-down of reactive oxygen and nitrogen species (ROS and RNS, definitions as explained in Ref. [10] and the footnotes below) or inadequate repair of the resulting oxidative damage [11,12]. The resulting accumulation of oxidatively modified biomolecules and dysregulation of cellular redox signaling can be used as a diagnostic tool, in the form of defined redox biomarkers, to assess the magnitude of oxidative damage and alterations in redox pathways [13]. The concept of oxidative stress as a trigger of disease and death dates back to the year 1956, when Harman in 1956 postulated the “free radical theory of aging” [14]. However, also more than 60 years later, we rarely find drugs in clinical use that are by definition acting via an antioxidant mechanism, thereby questioning the “free radical theory of aging” [15,16]. In contrast, most cardiovascular mainstay drugs such as AT_1-receptor blockers, angiotensin-converting enzyme inhibitors, statins, beta-blockers and many more possess potent pleiotropic antioxidant properties [17,18].

Although most large clinical trials dedicated to the therapeutic efficacy of classical antioxidants such as vitamins C and E failed to demonstrate a clear benefit for cardiovascular prognosis (for review see Refs. [19–21]), there is clear evidence that most cardiovascular diseases (CVD) are associated with or even triggered by oxidative stress [22,23]. This fact is also well supported by phenotypic changes in animal models of CVD with genetic deletion or overexpression of enzymes involved in the synthesis or degradation of ROS and RNS [18,19,24,25]. Further support for the oxidative stress concept comes from modern approaches using systems biology to identify therapeutic targets based on redox processes within a specific disease and systematic overviews of redox drug candidates in advanced clinical phase II–III trials [26,27]. Overall the discrepancy between the rather disappointing outcome of antioxidant trials and the established presence of redox biomarkers as well as drugable redox targets in CVD highlights the need of better knowledge on sources of ROS and RNS, their interaction, discrimination between beneficial (physiological) redox signaling and detrimental (pathophysiological) oxidative damage pathways [10]. This knowledge gap can be potentially filled by better characterization and application of redox biomarkers, which may be used for personalized or precision medicine approaches to identify patients with high burden of oxidative stress and to apply optimized redox therapeutic strategies [28], as already suggested for cancer and cardiovascular therapy in particular as well as pharmacotherapy guidance and validation of drug efficacy in general [29,30]. In the subsequent paragraphs we will discuss different approaches to identify and quantify ROS and RNS formation in CVD by their footprints in the form of redox biomarkers with a critical assessment of their specificity and prognostic value.

2 Throughout this report, the term ROS refers almost exclusively to hydrogen peroxide and superoxide anion radical since both species are involved in redox signaling and are formed by a number of enzymatic sources. Other ROS include the hydroxyl radical or singlet oxygen, which however have most likely no specific roles in redox signaling but rather contribute to unspecified oxidative damage and oxidative stress. A large number of organic peroxides (e.g. lipid peroxides) are also covered by the term ROS but these species are only marginally discussed in the present overview. Other ROS such as hypochlorite are also not in the focus of the present work.

3 Throughout this report, the term RNS refers almost exclusively to peroxynitrite, peroxynitrous acid and derived free radicals (e.g. hydroxyl radicals and nitrogen dioxide radicals). Peroxynitrite anion has a high specificity for activated thiols and readily reacts with carbon dioxide (the latter supports radical reactions). Peroxynitrous acid also has a high specificity for activated thiols but also reacts with transition metal complexes. RNS also comprises nitric oxide, which upon reaction with oxygen can form nitrogen dioxide radicals or nitration species such as NO_2 and has a high affinity for transition metal complexes such as iron(II), all of which contributes to the potent redox signaling properties of nitric oxide. However, in the context of “oxidation of biomolecules” the term RNS does not refer to nitric oxide due to its weak reactivity towards biomolecules in general.

2. Redox biomarkers

2.1. Sources of ROS/RNS and their major role in health and disease

Primary sources of ROS such as NADPH oxidases and the mitochondrial respiratory chain can generate secondary sources of ROS by oxidative conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO) or NO-producing endothelial nitric oxide synthase (eNOS) to the uncoupled superoxide-generating enzyme [31]. NADPH oxidases are highly specialized ROS producing protein complexes with almost no other biological function [21]. Mitochondria, under normal conditions, produce ROS as a byproduct of the excessive use of oxygen, transport of electrons from reducing factors along the respiratory complexes and escape of these electrons to molecular oxygen (up to 1% of the total flow of electrons by estimation) [32]. In pathological situations, e.g. upon ischemia/reperfusion (I/R) the (redox) activation of p66Shc or monoamine oxidases (MAO), activation of mitochondrial ATP-sensitive potassium channels and opening of the mitochondrial permeability transition pore (mPTP) can dramatically increase the formation and release of ROS from mitochondria [25]. Also, a ROS-induced ROS formation via redox-based amplification mechanisms was described [33,34]. ROS can exert beneficial and detrimental effects, which makes their therapeutic targeting so difficult and so far mostly unsuccessful (reviewed extensively in Ref. [10]). They can have protective effects via preconditioning-like mechanisms, kill pathogens during host defense or even contribute to essential cellular redox signaling (e.g. in cell differentiation, proliferation and migration). Vice versa, after exceeding a certain threshold, ROS have adverse effects including oxidative damage of various biomolecules, activation of inflammatory and cell death (e.g. apoptotic) pathways. Dysregulated redox signaling, e.g. by dysregulation of protein kinase pathways and calcium handling [35,36], epigenetic homeostasis [37–39], lipid metabolism [36] and vascular function [18,40], is counter-balanced by oxidatively activated antioxidant defense systems like NRF2/KEAP1 or hypoxia-inducible factor-1α (HIF-1α) that control multiple ROS/RNS-degrading enzymes (e.g. heme oxygenase-1 (HO-1), superoxide dismutases (SOD) or thiol-based reductases/peroxidases) [12,26,41]. An example of this kind of dysregulated redox signaling was established for peripartum cardiomyopathy [42]. Redox biomarkers should help to discriminate these fundamentally different properties of ROS/RNS in health and disease enabling the search for novel redox therapeutic drugs.

2.2. What information should redox biomarkers provide and what basic requirements should they fulfill?

As discussed in detail by Frijhoff and colleagues, redox biomarkers should fulfill the following minimal requirements and provide the following minimal level of information [43]: By definition of the WHO, a biomarker represents any substance, structure or functional parameter that can be measured in a biological sample or the whole organism with prognostic value regarding the progression/severity of a disease or the health outcome [44]. In order to add clarity to these criteria, a clinically useful biomarker should be specific for a certain disease (diagnostic), have prognostic value or reflect the disease state or in other words the health risk of an individual. This information will allow monitoring of the therapeutic efficacy of drugs in already diagnosed illness or help to choose the appropriate drug and initiate therapy in so far undiagnosed disease states. For routine use in the clinics, a biomarker should possess a sufficient stability during measurement and storage and should be easily accessible in widely obtainable tissues or preferably in blood. Finally, cost-effectiveness is a major criterion in modern health care systems and reproducibility on a large scale. Criteria for biomarkers in general and redox biomarkers in particular were further refined by Ghezzi and coworkers providing more useful definitions (e.g. for discrimination between passive and active biomarkers) [15,45].

Besides these minimal essential requirements a biomarker should...
Fig. 2. Redox pathways associated with putative biomarkers of oxidative stress [10,43]. The processes that lead to oxidative modifications of proteins, lipids and nucleotides are highly complex. Enzymes, such as XO, NOX and NOS, as well as organelles such as mitochondria, can produce ROS and RNS. ROS/RNS from primary sources can trigger secondary ROS sources (e.g. conversion of XDH to XO or uncoupling of eNOS) [31]. ROS can serve as substrates for other enzymes to generate additional types of ROS, such as the generation of HOCI from H$_2$O$_2$ by MPO. Importantly, some ROS sources are solely producing detrimental ROS (filled red color, e.g. NOX1/2/5), some have physiological functions but generate ROS as a side product or in the presence of certain substrates (dashed red line, e.g. LPO, Mito) or even produce mostly beneficial ROS (filled red color with red line, e.g. NOX4). Cellular systems and enzymes, including the SOD, GSH/GPx and Trx/Prx system, counterbalance the production of ROS (filled green color). In addition, increased levels of ROS activate the transcription factor Nrf2 via Keap1 to transcribe genes such as HO-1 that are involved in countering these ROS (filled green color). Oxidative stress affects cGMP signaling through its effects on nitric oxide (•NO) production, scavenging and on the •NO producing enzyme eNOS as well as the •NO receptor sGC (by oxidative heme depletion to the apo-sGC) and its target kinase PKG. In principle, all of these mentioned ROS producing, detoxifying and oxidatively modified enzymes and systems represent redox biomarkers as their expression or activity will provide information on the redox balance. ROS and RNS can be directly detected using specific probes (e.g. by chemiluminescence, fluorescence based HPLC or EPR-based methods) [46,57]. ROS and RNS also leave footprints in vivo in the form of classical oxidative stress markers such as oxidized amino acids in proteins or oxidized nucleotides in DNA/RNA as well as free or protein-bound reactive aldehydes from glucotoxicity and lipid peroxidation (filled grey color) [43]. In addition, functional readout of signaling pathways (e.g. redox-regulated kinases and calcium-handling [35,36], epigenetic [37–39] and lipidome profiles [36], P-VASP as the endpoint of the NO/cGMP signaling cascade and novel activity markers such as sNox2-dp or apo-sGC; all filled purple color) can significantly add to our understanding and identification of redox imbalances. For the sake of clarity, we could not show and discuss all existing ROS/RNS sources, species and targets in the present scheme, but focused on the most important ones according to the cited references. **Abbreviations:** AGE, advanced glycation end products; cGMP, cyclic guanosine monophosphate; EPR, electron paramagnetic resonance; GSH, glutathione; GPx, GSH peroxidase; GR, GSH reductase; H$_2$O$_2$, hydrogen peroxide; HO-1, heme oxygenase-1; HOCI, hypochlorous acid; LPO, lipooxygenases; MAO, monoamine oxidase; MPO, myeloperoxidase; NOS, nitric oxide synthase; NOX, NADPH oxidase; ONOO$^-$, peroxynitrite; PKG, protein kinase G; Prx, peroxiredoxin; P-VASP, phospho (Ser239)-vasodilator-stimulated phosphoprotein; RNS, reactive nitrogen species; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; sNox2-dp, soluble Nox2-derived peptide; SOD, superoxide dismutase; Trx, thioredoxin; TR, Trx reductase; u-eNOS, uncoupled eNOS; XDH, xanthine dehydrogenase; XO, xanthine oxidase. Reused from Ref. [43] and updated from Refs. [10,57,161] with permission under the terms of the Creative Commons Attribution Noncommercial License. Copyright © [43]; Published.
fulfill, it would be also desirable to learn more about the nature of ROS/RNS (helpful for approaches using specific ROS/RNS scavengers) as well as the cellular site and organ system of their formation (helpful to understand the pathophysiological role of ROS/RNS in the respective disease) [10]. However, for the simple conclusion that a certain disease is associated with excessive ROS and RNS formation (oxidative stress), their chemical identity is not required and accordingly sophisticated ROS and RNS detection techniques are not necessary but classical oxidative stress biomarkers (e.g. 3-nitrotyrosine (3-NT) or 4-hydroxynonenal (4-HNE)-positive proteins or 8-oxo-G-positive RNA) will suffice for qualitative assessment of oxidative stress [46]. This approach may also be enough for simple tests of redox drugs, where it will be sufficient to see whether the oxidative phenotype in diseased animals or challenged cells changes and the tested drug successfully suppresses oxidative stress mediated damage. These tests can be further refined by treatment with specific inhibitors of ROS/RNS sources (e.g. NOX inhibitors, XO inhibitors) or specific scavengers (e.g. SOD mimetics, mitochondria-targeted antioxidants), which will add to further mechanistic insights, even for classical oxidative stress marker-based assays.

2.3. Categories of redox biomarkers

Redox biomarkers can be separated into different categories as also defined in the past (Fig. 2) [10,43]. They comprise the classical oxidative stress markers that in most cases represent endproducts of oxidation reactions such as lipid peroxidation, oxidized amino acids (free or protein-bound), oxidized DNA/RNA bases (free or DNA/RNA-bound) and changes in thiol redox state (simplest example is reduced glutathione (GSH)/oxidized glutathione disulfide (GSSG) ratio). Another category of redox biomarkers relies on expression/activity of enzymes that are involved in the formation (e.g. NOX, XO) or breakdown (e.g. SODs, peroxiredoxins) of ROS/RNS. Also the modulation of reporter molecules for signaling cascades (e.g. phosphorylated vasodilator-stimulated phosphoprotein [P-VASP] as the endtarget of NO/cyclic guanosine monophosphate (cGMP) signaling) or concentrations of endogenous inhibitors (e.g. asymmetric dimethyl arginine as inhibitor of eNOS [47]) as well as eNOS uncoupling surrogates (e.g. S-glutathionylation of eNOS) can serve as redox biomarkers [48]. As these rather traditional redox biomarkers, at least if not a plethora of them is determined in the same sample, provide only limited mechanistic insights regarding ROS/RNS identity or pathophysiology and are considered not specific at all for a certain disease or comorbidity, we will here focus on the evaluation of their prognostic value and their additivity in the presence of different comorbidities in human disease studies. Finally, redox profiling represents an emerging technique to obtain a complete picture of the redox changes that characterize a certain disease. Examples are redox lipidomics that characterizes the landscape of oxidized fatty acid products by means of mass spectrometry, kinase activity networks using phosphoproteomics or epigenetic profiling. These emerging techniques may yield specific redox profiles of certain disease and also provide detailed mechanistic insights.

2.4. Direct detection of ROS and RNS formation using fluorescence, chemiluminescence or UV/Vis absorption

The direct measurement of ROS and RNS formation represents a redox biomarker, although not easily applicable in large cohort studies as the assays need to be applied to freshly harvested tissue or biological fluid samples (at least the incubation of the biological samples with the probes). These direct ROS and RNS assays can provide information on the involved reactive species and are mostly based on fluorescence or chemiluminescence dyes combined with HPLC- or plate reader-based detection/quantification methods as well as on UV/Vis photometric assays. These techniques will not be discussed further here as they were reviewed in detail in the past. The classical “old” assays such as hydroethidium-dependent fluorescence microtopography in tissue cryosections to assess vascular ROS formation, lucigenin-enhanced chemiluminescence in tissue membrane fractions to quantify NADPH oxidase activity and luminol derivative (L-012)-enhanced chemiluminescence in whole blood to determine oxidative burst of leukocytes were critically discussed extensively in the past [46]. The measurement of oxidative burst by L-012-enhanced chemiluminescence in whole blood was also successfully applied in clinical studies and correlated with the walking distance as well as critical limb ischemia in patients with peripheral artery disease [49,50]. The combination of advanced HPLC-based fluorescence assays for the quantification of ROS and RNS formation was described for cell culture [51] that can be also used for specific detection of cellular superoxide generation [52]. In addition there are excellent reviews available discussing the detection of ROS and RNS formation in biological samples [53,54], including statements of the American Heart Association defining first and second choice assays [55].

Other experimental techniques comprise ROS-induced formation of microbubbles that are formed from liposome-encapsulated allylhydrazine, a liquid compound that yields nitrogen and propylene gas, which may be detected by ultrasound by in vivo application to animals [56] and in the future potentially in humans [reviewed in Ref. [9]]. Also, L-band electron paramagnetic resonance (EPR) spectroscopy in combination with the most advanced spin traps represents a cutting-edge technique for the in vivo detection of ROS and RNS, which is currently translated from animal to human application [57]. Furthermore, the combination of different techniques such as immuno-spin trapping as reported for trapping of thyl radicals in proteins or other radicals in DNA by 5,5-dimethyl-1-pyrroline N-oxide (DMPO) and formation of stable spin-adducts with subsequent immunological detection of DMPO-bound proteins or DNA molecules is already used for the detection of ROS and RNS formation, or more precisely their footprints in biological samples [58]. Additionally, the targeting of specific chemiluminescent or fluorescent dyes and/or ROS and RNS scavengers by linking them to site specific antibodies or loading them into nanoparticles might provide the next step in oxidative stress and redox biology research and may be used in humans in the near future [reviewed in Refs. [57,59]]. For preclinical research also genetically encoded fluorescent enzymatic probes, e.g. HyPer for hydrogen peroxide detection [60,61] and gE NOps for nitric oxide [62,63], will in the future allow the highly specific and timely as well as spatially resolved monitoring of ROS and RNS formation in isolated cells. More recently, the technique of genetically-encoded redox sensors was also translated to the animal level allowing the in vivo monitoring of redox changes in organs, even distinguishing between cytosolic and mitochondrial ROS/RNS formation by encoding a cytosolic or mitochondrial glutaredoxin/green fluorescent protein construct (Grx1-roGFPP2) [64,65]. Redox changes in tissues of these animals can be even conserved by alkylolation of reduced thiols in the Grx1-roGFPP2 construct for later “redox histology” using fluorescence microscopy [64,66].

3. Classical “oxidative stress” biomarkers in human cardiovascular disease

Mounting evidence indicates classical biomarkers of oxidative stress to be a prominent feature and suggestive of disease severity in several CVD phenotypes by displaying increased expression of these markers in CVD compared with control groups. Importantly, these markers are also
partly predictive of future cardiovascular events and mortality in initially healthy subjects after adjustment for established cardiovascular risk factors, thereby demonstrating an independent crucial role of oxidative stress in the development and progression of CVD and mortality. However, future studies should address the incremental value of oxidative stress markers beyond established cardiovascular risk factors for the prediction of incident cardiovascular events and related outcomes.

3.1. Lipid peroxidation products 8-isoprostane, 4-hydroxynonenal and malondialdehyde

Malondialdehyde (MDA) is one of the best studied and most abundant (10–20 μM in plasma) endproducts of lipid peroxidation chain reactions [10,43]. Other members of this class of redox biomarkers comprise peroxidation products of cholesterol, phospholipid and polyunsaturated fatty acids including ketones such as 4-HNE [67,68], alcohols such as isoprostanes [69], hydroperoxides such as 1-palmitoyl-2-(5′-oxo-valeroyl)-sn-glycero-3-phosphocholine, oxidized low-density lipoprotein (LDL) [70] and cyclic endoperoxides. Polyunsaturated fatty acids (free or in phospholipids) containing unconjugated olefinic bonds are easily oxidized by ROS/RNS and derived free radicals [71]. Commonly reported examples of oxidized cholesterol (oxysterols) include 6-cholesten-5α- and 25-hydroxycholesterol, 7-dehydrocholesterol and 7-ketocholesterol, 7-dehydrocholesterol and 25-hydroxycholesterol [72]. We here focus on 8-isoprostane, 4-HNE and MDA as they are most widely used in the clinical routine, fairly specific ELISAs are commercially available and their formation mechanism is fully characterized [10,43,67]. The gold standard for detection of these products are gas and liquid chromatography in combination with mass spectrometry [73], which can at least be used to validate the results obtained with ELISA in selected samples. Of note, due to their reactivity 4-HNE and MDA (and other reactive lipid peroxidation products) can bind to thiol or amino groups in proteins (or other biomolecules) and thereby modulate the activity of enzymes and may act as toxifiers in disease pathomechanisms or confer redox hormesis via electrophilic activation of antioxidant defense systems. As a note of caution, we would like to mention that MDA and 4-HNE can also be formed by ROS-independent mechanisms such as the fragmentation of prostaglandin endoperoxide (PGH₂) or diendoperoxide (generated by oxygenation of arachidonic acid by cyclooxygenase-2 and 5-lipoxygenase) during platelet aggregation or inflammation [74].

A nested case-cohort study in postmenopausal women by Roest et al. showed that elevated levels of urinary 8-isoprostaglandin F2α (8-isopGF2α) were associated with increased cardiovascular mortality due to coronary artery disease (CAD) (141 deaths) and stroke (109 deaths) after a follow-up of 18 years [75]. An odds ratio (OR) of 1.8 (95% CI 1.1–3.1) was observed for the highest quartile of 8-isopGF2α levels (Fig. 3A), independent of age, while further adjustment for systolic blood pressure, history of CVD, diabetes, smoking, and body mass index did not attenuate this association. A more recent meta-analysis from 2017, aimed at classifying oxidative damages across 50 human health conditions including 242 studies, displayed small increases in 8-isopGF2α levels for hypertension (standardized mean difference (SMD) 0.6), chronic heart failure (SMD 1.7), CAD (SMD 0.4), stroke (SMD 1.4) compared to healthy subjects with cardiovascular disease patients.

Fig. 3. Prognostic value of classical oxidative stress markers for cardiovascular risk. (A) Association of markers of lipid peroxidation (isoprostane [8-iso-PGF2α] and malondialdehyde [MDA]) [75,83,85], nitro-oxidative stress (3-nitrotyrosine [3-NT]) [96], oxidative mitochondrial DNA damage (8-hydroxy-deoxyguanosine [8-OhdG]) [120] and derivatives of reactive oxygen metabolites (d-ROMs) [125] with cardiovascular risk in the comparison of healthy subjects with cardiovascular disease patients. (B) Associations of d-ROM levels and total thiol levels (TTL) with cardiovascular disease-specific mortality (adjusted for age and sex). d-ROMs groups (Carratelli Units): reference, <340; T1, 341–400; T2, 401–500; T3, >500. Graph was generated from tabular data in Ref. [124] with permission and reused from Ref. [48]. Copyright © 2015, Schöttker et al. * indicates significant differences (p < 0.05) versus reference groups.
correlations of 8-iso-PGF2α with New York Heart Association (NYHA) classification stages and left ventricular ejection fraction, respectively [78]. Contrarily, a study from Woodward et al. found no association between 8-iso-PGF2α levels and risk of fatal or non-fatal CAD (OR 1.08, 95% CI 0.91–1.29 per 1-standard-deviation (SD) increase in 8-iso-PGF2α levels) for the comparison of 227 coronary cases and 420 control subjects [79].

Nakamura et al. showed 5-fold increased 4-HNE levels in 23 patients with dilated cardiomyopathy compared with 13 control subjects with normal cardiac function [80]. The treatment of 11 subjects for 9 ± 4 months with carvedilol (22 ± 8 mg/d), a beta-blocker with antioxidant capacity, reduced 4-HNE levels by 40% along with functional amelioration of heart failure symptoms. In a study by Mak et al. comparison of 8 patients with chronic heart failure with 8 age-matched patients with normal left ventricular function revealed elevated levels of total aldehydes including 4-HNE in heart failure patients [81]. Barrios et al. reported increased 4-HNE levels in 22 patients with acute MI compared with 20 control subjects without presence of acute CAD. Infarct size was strongly correlated with 4-HNE levels [82].

In 774 chronic heart failure patients, MDA was a significant predictor of death and a combined endpoint of death and heart transplantation at one-year follow-up [83]. After adjustment for a range of clinical and laboratory variables, elevated levels of serum MDA were associated with a two-fold increased risk of death (hazard ratio (HR) 2.103, 95% CI 1.330–3.325) as well as a two-fold increased risk of the combined endpoint (HR 2.000, 95% CI 1.366–2.928 per 1 μmol/L). For the comparison of the highest vs. lowest quartile of baseline MDA levels the relative risk (RR) of death was 3.64 (95% CI, 1.917–6.926) and the RR for the combined endpoint was 2.71 (95% CI 1.551–4.739) (Fig. 3A). In line with this, Radovanovic et al. reported that high plasma MDA levels (>8 μmol/L) in 120 chronic heart failure patients independently increased risk of death in median 13-month follow-up [84]. Patients with MDA levels above the cut-off had an eight times higher risk of death. A large prospective study of 634 patients with stable CAD from the PREVENT trial was able to demonstrate that serum MDA increased the risk of cardiovascular events [85]. For the comparison of the highest vs. lowest quartile of MDA, a RR of 3.30 (95% CI 1.47–7.42) for major vascular events (fatal/non-fatal MI and stroke) (Fig. 3A), a RR of 4.10 (95% CI 2.55–6.60) for non-fatal vascular events (unstable angina) and a RR of 3.84 (95% CI 2.56–5.76) for major vascular procedures (percutaneous interventions and coronary artery bypass grafting) were observed.

A meta-analysis of 12 observational studies aimed at investigating the association between oxidized LDL and risk of atherosclerotic CVD revealed an overall effect size of 1.79 (95% 1.56–2.05) [86]. Among 1371 patients with acute MI, increased levels of oxidized LDL were associated with a combined endpoint of death due to CVD, occurrence of unstable angina pectoris and MI at 6-month follow-up (HR 1.66, 95% CI 1.07–2.57 for highest vs. lowest tertile) [87]. Another study has shown a 215% (95% CI 47–576) increased risk of a major adverse coronary event in 246 patients with CAD in dependence of oxidized LDL levels (for highest vs. lowest quartile) [88]. An association between oxidized LDL and cardiovascular events was also reported in a study of 425 patients with acute coronary syndrome by demonstrating an HR of 2.88 (95% CI 1.93–4.32 per 1 mmol/L increase in oxidized LDL) for acute MI recurrence or acute coronary syndrome-related death at 5-year follow-up [89].

Ethylene in the breathing air of patients undergoing cardiopulmonary bypass surgery allowed real-time monitoring of endogenous lipid peroxidation in the intraoperative setting and was indicative of regional myocardial ischemia and reperfusion [90]. The authors suggest ethylene monitoring as a non-invasive method of risk stratification and prevention of intraoperative complications during cardiopulmonary bypass surgery. However, so far this method requires expensive instruments (mass spectrometry) that may not be affordable and applicable in every small hospital.

3.2. 3-Nitrotyrosine as a footprint of peroxynitrite in vivo formation (or inflammatory activation of the myeloperoxidase/hydrogen peroxide/ nitrite pathway) and other NO-related markers

The major nitro-oxidative protein modifications elicited by peroxynitrite (ONOO⁻) are S-glutathiolation and S-oxidation of cysteine as well as nitration of tyrosine residues. Other modifications are zinc finger oxidation, methionine sulfoxidation and nitration of tryptophan residues by ROS as well as RNS [10,43]. We here focus on 3-NT as there is substantial clinical evidence for its association with disease pathogenesis, fairly specific EIAs are commercially available and its formation mechanism is well understood. Importantly, most other oxidative protein modifications are not specific for ONOO⁻. Regarding a summary of the clinical evidence for other protein-based oxidative stress markers, also including protein carbonyl groups, we refer to previous overviews [10,43]. The immunological detection methods can be validated by mass spectrometry-based methods. Importantly, also 3-NT can act as a toxifier in disease pathogenesis by triggering autoimmune reactions (IgGs against 3-NT) that also correlate with CAD health outcomes [91], inactivating important ROS scavenging and vasodilator-producing enzymes such as Mn-SOD [92] and prostacyclin synthase [93], or aggravating the prothrombotic effects of fibrinogen [94,95].

A case-control study (100 cases with established CAD and 108 controls with no clinical history or symptoms suggestive of CAD) by Shishehbor et al. aimed at investigating the association of systemic 3-NT levels and the presence of CAD [96]. The authors demonstrated that in patients with CAD, 3-NT levels were markedly higher compared with control subjects (median values, 9.1 vs. 5.2 μmol/mol). Likewise, prevalence of CAD increased with higher 3-NT quartiles (26% in the lowest vs. 58% in the highest quartile). After multivariable adjustment for the Framingham Global Risk Score and levels of C-reactive protein, increased 3-NT levels were associated with higher odds of CAD (OR 6.1, 95% confidence interval (CI) 2.6–14.0 for highest vs. lowest quartile) (Fig. 3A). Moreover, statin therapy in 35 participants (atorvastatin 10 mg/d) for 12 weeks reduced 3-NT levels by 25%. Concordantly, Pirro et al. were able to demonstrate that the combination of a 4-week rosuvastatin therapy (10 mg/d) and low-fat diet was associated with a pronounced decrease in 3-NT levels as well as arterial stiffness, a well-established marker of CVD risk, in 35 patients with primary hypercholesterolemia (vs. 36 patients with a low-fat diet only) [97]. A study of 120 prediabetic patients by Chu et al. demonstrated increased levels of 2-h 3-NT levels after 75g oral glucose tolerance test in patients with concomitant CAD compared with patients having no CAD [98]. After multivariable adjustment for risk factors and glucose tolerance status, 2-h 3-NT levels in the highest quartile (OR 3.1, 95% CI 2.2–5.3 vs. lowest quartile) remained independently associated with the presence of CAD. Conversely, a recent study by Quidin et al. found that 3-NT levels were not predictive of mortality rates during a follow-up period of four years in a cohort of 342 patients with CAD [99].

Also, other products of nitric oxide-dependent reactions or even NO gas itself were used in the past or may be used in the future to test their diagnostic and prognostic value in CVD. Although nitrotyrosopan derivaties were not used so far as markers of nitro-oxidative stress in the clinical setting, their measurement may provide superior insights in the RNS being formed and analytical methods to determine their levels in tissues and biological fluids have been established [100]. Another emerging marker of nitro-oxidative stress that was not applied in clinical studies so far is the nitroprotein 3-nitrotyrosine (3-NT) (8-nitro-cGMP), which itself can act as an electrophile and contribute to cellular redox regulation [101,102]. Nitrated fatty acids represent another footprint of RNS reactions in vivo, display potent electrophilic properties and contribute to cellular redox signaling [103,104]. Of note, nitro-fatty acids could not only serve as redox biomarkers but also represent potent therapeutic compounds [105] and analytical methods are in place to detect and quantify these species in biological samples [106]. Recently, the urinary nitrolipidome was quantified using high
resolution mass spectrometry with fragmentation analysis [107].

The use of gaseous NO in exhaled air as a redox biomarker is inconclusive so far, as increased NO may be indicative of higher iNOS activity and an inflammatory phenotype, but vice versa may also mirror improved eNOS function and higher endogenous vasodilatory NO production. The exhaled NO concentrations showed a moderate trend of being lower in patients with stable CAD as compared to healthy subjects and was further decreased in both groups among active smokers [108]. Of note, physical exercise decreased exhaled NO concentrations in CAD patients but had only minor effects in healthy subjects, which may indicate dysregulated exercise-induced eNOS activation in the setting of CAD. The link between exhaled NO concentrations and endogenous NO production was supported by a dramatic increase in NO in the breathing air and plasma cGMP levels after injection of L-arginine in patients with pre eclampsia [109]. Therapy of hypertensive patients with the calcium antagonist amlodipine increased exhaled levels of NO in response to exercise [110] and exhaled NO concentrations correlated with exercise intolerance in patients with heart disease [111]. Whereas the measurement of exhaled NO levels as a diagnostic and prognostic tool for assessment of cardiovascular risk is still in its infancy, the application of this assay was already tested on a broader basis in patients with asthma and chronic obstructive pulmonary disease (COPD) to determine the inflammatory status in the lung [112].

The plasma or serum levels of the NO degradation products nitrite and nitrate have only limited diagnostic and prognostic value as they are largely affected by dietary intake of these anions, especially by certain vegetables. However, in 2885 Framingham Offspring Study subjects, nitrate levels were associated with increased all-cause mortality (HR 1.21, 95% CI 1.03–1.40 per unit increase) at median follow-up of 17.3 years after adjustment common risk factors, whereas no association was observed for incident CVD (HR 1.08, 95% CI 0.89–1.31) [113]. Importantly, in an analysis of 2443 subjects without CVD at baseline, nitrate/nitrite levels were predictive of incident CVD (OR 1.35, 95% CI 1.01–1.80), further showing that nitrate/nitrite measurement added to a better reclassification of individuals at risk of CVD beyond classical cardiovascular risk factors [114].

3.3. Oxidative DNA/RNA damage

Nucleotide oxidation is a highly abundant DNA modification [115] and represents a pre-mutagenic lesion that induces CG/TA transversion mutations, which can also contribute to cancer [116]. The guanine moiety is particularly prone to oxidation under formation of 8-hydroxy-2-deoxyguanosine (8-OHdG) or 8-OHG (8-oxoGuo) and can be used as a marker of DNA and RNA oxidation by Fenton chemistry-derived hydroxyl radicals or peroxynitrite. Levels of free 8-OH(d)G can be measured upon DNA/RNA digestion and hydrolysis from tissues or directly in urine and serum/plasma [10,43]. Whereas commercial ELISAs are available but critically discussed [117], the gold standard for measurement is based on mass spectrometry methods.

A meta-analysis on the association between 8-OHdG levels and CVD (CAD, stroke, peripheral artery disease and carotid atherosclerosis) including five case-control studies and nine prospective studies (810 CVD patients and 1106 control subjects) showed higher levels of 8-OHdG in CVD patients compared with controls (SMD 1.04, 95% CI 0.61–1.47) [118]. In good agreement, a further meta-analysis of six studies (446 heart failure patients and 140 controls) from the same author group showed higher levels of 8-OHdG in heart failure patients than in controls (SMD 0.89, 95% CI 0.68–1.10) with a progressive increase for higher NYHA class [119]. A recent study by Wang et al. from 2020 aimed to investigate whether 8-OHdG may contribute to the incidence of CAD in 1920 patients with type 2 diabetes mellitus [120]. After multivariable adjustment, the authors reported elevated levels of 8-OHdG to be cross-sectionally related to increased odds of obstructive CAD (OR 1.38, 95% CI 1.24–1.52), higher number of diseased vessels with ≥ 50% stenosis (OR 1.29, 95% CI 1.19–1.41), modified Gensini score (OR 1.28, 95% CI 1.18–1.39) and higher levels of C-reactive protein (β 0.18, 95% CI 0.06–0.31). Importantly, increased levels of 8-OHdG were associated with a 59% higher risk of a major adverse cardiovascular and cerebral event in 701 diabetic patients subjected to coronary revascularization at one-year follow-up (hazard ratio (HR) 1.59, 95% CI 1.33–1.90 per 1-SD increase in 8-OHdG levels) (Fig. 3A). A study by Liu et al. found the extent of vascular complications to be associated with elevated RNA damage by measuring 8-HOG (8-oxoGuo) levels in 633 type 2 diabetes mellitus patients compared to 683 controls [121]. Levels of 8-HOG were highest among type 2 diabetes mellitus patients with vascular complications followed by type 2 diabetes mellitus patients without vascular complications and healthy controls, increasing overall with age. In good agreement, in 936 patients with type 2 diabetes mellitus, higher levels of 8-HOG were associated with increased risk of diabetes-related mortality including death from MI, stroke, and peripheral vascular disease at follow-up (HR 1.72, 95% CI 1.11–2.66 for quartile 4 vs. quartile 1) [122]. Furthermore, Broedbaek et al. demonstrated that 8-HOG levels predicted incident CVD in 1381 type 2 diabetes mellitus patients [123].

3.4. Other, less specified classical redox biomarkers such as ROM or TTL

In a meta-analysis from Schöttker et al. serum derivatives of reactive oxygen metabolite (D-ROM) and total thiol (TTL) levels were measured to determine their impact on all-cause and CVD mortality in 10,622 subjects from population-based cohorts from Germany, Poland, Czech Republic and Lithuania [124]. The results revealed that both D-ROM and TTL were independently associated with all-cause (D-ROM: RR 1.20, 95% CI 1.12–1.29 per 1-SD increase; TTL: RR 1.12, 95% CI 1.04–1.21 per 1-SD decrease) and CVD mortality (D-ROM: RR 1.30, 95% CI 1.12–1.51 per 1-SD increase; TTL: RR 1.25, 95% CI 1.09–1.45 per 1-SD decrease) after adjustment for established risk factors (Fig. 3B). A pooled analysis of the same cohorts from Xuan et al. also indicated D-ROM levels to be associated with MI (OR 1.21, 95% CI 1.05–1.40 per 100 Carr units increase) (Fig. 3A) and stroke incidence (OR 1.17, 95% CI 1.01–1.35), while TTL levels were only associated with the incidence of stroke (OR 0.79, 95% CI 0.63–0.99 for quartiles 2–4 vs. quartile 1) [125]. Masaki et al. confirmed the prognostic value of D-ROM by showing that high levels of D-ROM (highest vs. lowest quartile) in 265 patients with treated CVD were associated with increased risk of all-cause mortality (HR 10.791, 95% CI 1.032–112.805) and cardiovascular events (HR 2.651, 95% CI 1.138–6.177) at follow-up [126].

4. Redox-regulated microRNAs in human cardiovascular disease

Beyond the classical oxidative stress biomarkers, epigenetic profiling may represent a promising redox diagnostic strategy in the future, especially as the costs per assay continue to decrease. A group of naturally occurring, small and noncoding RNAs, microRNAs (miRNAs), has been identified as regulators of gene expression that are known to regulate approximately 30% of human genes [127,128]. In the clinical setting, miRNAs are regarded as advantageous biomarkers as they can be easily obtained from peripheral blood samples and are stable at room temperature as well as after being frozen and thawed [129]. A number of miRNA expression levels were shown to change in response to oxidative stress via their respective redox interaction partners comprising NRF2 and SIRT1, both detailed in this review, as well as NF-κB [130,131]. Due to the sheer number of miRNAs described in oxidative-stress-induced heart disease, a selection of prominent miRNAs involved in the onset and progression of CVD will be presented in the next section.

While miRNAs have been recognized as a diagnostic tool which still has to undergo robust clinical evaluation, the future may see multiple ways to therapeutically influence miRNA expression. Antagomirs, oligonucleotides designed to bind and inhibit miRNA [132], as well as miRNA mimics, which are designed to therapeutically re-establish miRNA levels [133], are currently being investigated within clinical
Table 1

| Biomarker | Study type | Quality level | Sampling/method of detection | Major outcome | Reference |
|-----------|------------|---------------|-------------------------------|---------------|-----------|
| 3-NT      | Case-control study (100 CAD patients, 108 CTR) | +++ | Plasma/stable isotope dilution liquid chromatography–electrospray ionization tandem mass spectrometry-based method using an ion trap mass spectrometer | Increased 3-NT in CAD patients. OR of 4.4 (95% CI 1.8–10.6 for highest vs. lowest quartile) for CAD. | [96] |
| 3-NT      | Prospective cohort study (342 CAD patients) | +++ | Serum/colorimetric ELISA | No relationship between 3-NT and mortality rates during 4-year follow-up. | [99] |
| 3-NT      | Clinical trial (120 prediabetic patients) | +++ | Plasma/ELISA | 2-h 3-NT after 75 g oral glucose tolerance tests is related to the presence of CAD (OR 3.1, 95% CI 2.2–5.3 for highest vs. lowest quartile). | [98] |
| 3-NT      | Animal study | + | Immunohistochemical analysis of rat hearts | Increased 3-NT after ischemia/reperfusion injury in rat hearts. | [243] |
| 3-NT      | Animal study | + | Dot blot method analysis of rat hearts | Increased 3-NT after MI in rats. | [244] |
| HNE       | Clinical trial (23 patients with dilated cardiomyopathy, 13 CTR) | ++ | Immunohistochemical analysis of endomyocardial biopsies | 5-fold increased HNE in cardiomyopathy patients. Treatment with carvedilol reduces HNE by 40%. | [80] |
| HNE       | Clinical trial (22 patients with acute MI, 20 CTR) | ++ | Plasma/high-performance liquid chromatography | Increased HNE in MI patients. Infarct size strongly correlates with HNE. | [245] |
| HNE       | Animal study | + | High-performance liquid chromatography analysis of rat hearts | Generation and release of HNE in isolated perfused hearts of normotensive and spontaneously hypertensive rats with cardiac hypertrophy and signs of HF during ischemia and reperfusion. | [246] |
| HNE       | Animal study | + | Immunohistochemical and image analysis of rat hearts | Presence of HNE-modified proteins in ischemic rats hearts after transplantation and reperfusion. | [247] |
| 8-iso     | Case-cohort study (250 cases of death due to CAD and stroke, 142 CTR) | +++ | Urine/liquid chromatography/tandem mass spectrometry | Increased 8-iso PGF2α predicts cardiovascular mortality in postmenopausal women (OR 1.8, 95% CI 1.1–3.1 for highest vs. lowest quartile). | [75] |
| PGF2α     | Meta-analysis (242 studies) | ++++ | Mixed (plasma, urine, sputum) | Increased 8-iso PGF2α in patients with hypertension (g 0.4, 95% CI 0.2–0.6), chronic HF (g 1.4, 95% CI 1.1–1.7), CAD (g 0.4, 95% CI 0.3–0.5) and ischemic stroke (g 1.2, 95% CI 1.0–1.4). | [76] |
| 8-iso     | Animal study | + | Serum/enzyme immuno-assay | Increased 8-iso PGF2α in the myocardium of rabbits with HF. 8-iso PGF2α correlates with cardiac function. | [248] |
| PGF2α     | Animal study | + | Plasma and myocardial tissue/ELISA | Increased 8-iso PGF2α in rats with acute myocardial ischemia. | [249] |
| 8-OHdG    | Meta-analysis (14 studies) | ++++ | Mixed (urine and blood) | Increased 8-OHdG in CVD patients (SMD 1.04, 95% CI 0.61–1.47). | [118] |
| 8-OHdG    | Meta-analysis (6 studies) | ++++ | Mixed (urine and plasma) | Increased 8-OHdG in HF patients (SMD 0.89, 95% CI 0.68–1.10). | [119] |
| 8-OHdG    | Animal study | + | Immunohistochemical and image analysis of rat hearts as well as DNA analysis using ELISA | 8-OHdG is related to cardiac dysfunction after ischemia/reperfusion injury in isolated rat hearts. | [250] |
| 8-OHdG    | Animal study | + | Immunohistochemical myocard analysis | Left ventricular dilatation and systolic dysfunction is associated increased 8-OHdG in rabbits after MI. | [251] |
| MDA       | Prospective cohort study (774 chronic HF patients) | +++ | Serum/Ohkawa’s method | Increased MDA predicts death in chronic HF patients (RR 3.64, 95% CI 1.917–6.926 for highest vs. lowest quartile). | [83] |
| MDA       | Prospective cohort study (634 stable CAD patients) | +++ | Serum/TBARS | Increased MDA predicts cardiovascular events in patients with stable CAD (RR 3.30, 95% CI 1.47–7.42 for major vascular events, RR 4.10, 95% CI 2.55–6.60 for non-fatal vascular events and RR 3.84, 95% CI 2.56–5.76 for major vascular procedures). | [85] |
| MDA       | Animal study | + | Plasma/ELISA | Increased MDA after myocardial injury induced by sepsis in rats. | [252] |
| MDA       | Animal study | + | Immunohistochemical analysis of cardiac tissues | Increased MDA after HF induced by left anterior descending coronary artery ligation rats. | [253] |
| D-ROM     | Meta-analysis (10,622 subjects) | ++++ | Serum/FRAP assay | Increased D-ROM predicts all-cause (RR 1.20, 95% CI 1.12–1.29 per 1-SD increase) and CVD mortality (RR 1.30, 95% CI 1.12–1.51) in 10,622 subjects from population-based cohorts. | [124] |
| D-ROM     | Pooled case-control study (476 cases of MI and 2280 CTR, 454 cases of stroke and 2270 CTR) | +++ | Serum/FRAP assay | Increased D-ROM predicts MI (OR 1.21, 95% CI 1.05–1.40 per 100 Carr units increase) and stroke (OR 1.17, 95% CI 1.01–1.35). | [125] |
| D-ROM     | Animal study | + | Plasma/photometric test | Increased D-ROM in the myocardium of hypoxic rats. | [254] |
| D-ROM     | Animal study | + | Serum/fras4 test | (continued on next page) | [255] |
Table 1 (continued)

| Biomarker | Study type | Quality level | Sampling/method of detection | Major outcome | Reference |
|-----------|------------|---------------|-------------------------------|---------------|-----------|
| TTL       | Meta-analysis (10,622 subjects) | ++++ | Serum/FRAP assay | Increased D-ROM is related to ventricular remodeling and arrhythmia in rats. | [124] |
| TTL       | Pooled case-control study (476 cases of MI and 2380 CTR, 454 cases of stroke and 2270 CTR) | ++++ | Serum/FRAP assay | Increased TTL predicts all-cause (RR 1.12, 95% CI 1.04–1.21 per 1-SD decrease) and CVD mortality (RR 1.25, 95% CI 1.09–1.45) in 10,622 subjects from population-based cohorts. | [125] |
| TTL       | Animal study | + | Serum/spectrophotometry | Induction of MI in rats is associated with a decrease in TTL. | [256] |
| TTL       | Animal study | + | Cardiac tissue/spectrophotometry | Induction of MI in rats is associated with a decrease in TTL. | [257] |

**: Medium quality (animal study), ++: Good quality (clinical trial/cohort study <100 subjects), +++: Strong quality (clinical trial/cohort study >100 subjects), ++++: Very strong quality (large scale meta-analysis), 3-NT: 3-nitrotyrosine, CAD: Coronary artery disease, CTR: Controls, OR: Odds ratio, CI: Confidence interval, TBARS: Thiobarbituric Acid Reactive Substances, FRAP: Ferric Reducing Antioxidant Power, fras4: Free Radical Analytical System 4, HNE: 4-Hydroxynonenal, MI: Myocardial infarction, HF: Heart failure, 8-iso PGF2α: 8-iso-prostaglandin F2α, g: standardized mean difference - Hedges’ g, 8-OHdG: 8-Hydroxy-2-deoxyguanosine, CVD: Cardiovascular disease, MDA: Malondialdehyde, RR: Relative risk, D-ROM: Derivatives of reactive oxygen metabolite, SD: Standard deviation, TTL: Total thiol.

Table 2

Selected microRNAs in human CVD.

| miRNA | CVD | Clinical use | Molecular mechanism | Reference |
|-------|-----|--------------|---------------------|-----------|
| 199   | CAD | Associated with MACE reduction | SIRT-1 induction | [140] |
| 21    | Heart failure | Screening parameter | Upregulation of natriuretic peptide b | [147, 148] |
| 1254 and 1306-5p | Heart failure | Association with mortality | Unknown | [145] |
| 26b-5p, 660-5p, and 320a | STEMI | MACE prediction | Unknown | [141] |
| 107, 22-3p, and 146a5p | AF | MACE prediction | Unknown | [160] |

MACE: major adverse cardiovascular events; SIRT-1: sirtuin 1; STEMI: ST-elevation myocardial infarction; AF: atrial fibrillation.

trials [134]. Notably, none of the trials involve patients suffering from CVD.

MicroRNAs display a huge potential as a screening marker in CVD while at the same time suffering from an enormous gap of evidence. On the one hand, certain miRNAs were shown independently to predict cardiovascular events in certain pathological conditions (Table 2) while on the other, only a fraction of them has so far been investigated regarding their molecular targets. Further research on the molecular mechanisms of miRNAs in CVD seems warranted as their redox-sensitivity can be assumed from the available data [135], from their own contribution to ROS/RNS formation [136] and from the fact that their levels change in diseases that are associated with oxidative stress.

4.1. Coronary artery disease and miRNAs 24, 92a and 199a

miRNA-24 is highly expressed in the vasculature [137] and is upregulated in response to vascular injury which in turn indirectly activates the NRF2-pathway by regulating KEAP1 resulting in an overall protective effect [138]. While miRNA-92a overexpression leads to impaired endothelial function by way of reducing heme oxygenase-1 (HO-1) expression, thus promoting the formation of oxidative stress in a murine model for diabetic vasculopathy [139], miRNA-199a expression was found to be lowered in patients with CAD [140]. In these patients, who had undergone coronary artery bypass surgery, miRNA-199a expression levels were found to be reduced in myocardial tissue in those who would be less likely to suffer from major adverse cardiac and cerebrovascular events over the course of the three years following surgery [140]. This predictive effect was attributed to the upregulation of SIRT1 in response to lowered miRNA-199a levels.

4.2. Myocardial infarction and miRNAs 26b-5p, 660-5p and 320a

In patients presenting with ST-segment elevation MI (STEMI), a study by Jakob and colleagues found three miRNAs, 26b-5p, 660-5p and 320a, which were associated with cardiac death or recurrent MI within the next year [141]. In this study, miRNA levels in blood samples were shown to independently predict MACE while their predictive value was improved via combination with the clinically used GRACE score. While the miRNAs associated with cardiovascular events in this study are known to be involved in the development of cardiac hypertrophy [142] and cardiomyocyte apoptosis [143], no experimental data are available regarding their redox-sensitivity.

4.3. Heart failure and miRNAs 1254,1306 and 21

Amongst the myriad of microRNAs screened in the serum of patients suffering from heart failure (reviewed in detail by Zhou and colleagues [144]), miRNAs 1254 and 1306 have recently been identified as prognostic markers in the blood of approximately 2200 patients [145]. While in this study, higher levels of miRNAs 1254 and 1306 were associated with the risk for all-cause mortality and hospitalization due to heart failure, the mechanism behind this effect remains unknown [145]. MiRNA-1254 was also predictive of ventricular remodeling in patients with ST-segment-elevation MI [146]. MiRNA-21 was found to be upregulated in murine models for heart failure by Vettori and colleagues [147] as well as in patients suffering from heart failure [148-150], subclinical diabetic cardiomyopathy [151] and diastolic dysfunction [152]. MiRNA-21 is upregulated in multiple types of human cancer [153] and cell-growth in general, and consequently linked to cardiac hypertrophy [154] and vascular neointimal lesion formation [155]. MiRNA-21 was also implicated in suppression of angiogenic progenitor cell function in CAD patients via inhibition of eNOS by asymmetric dimethyl arginine [156]. Of note, treatment with the antagonimer anti-miRNA-21 prevented myocardial damage and normalized cardiac function in a pig model of I/R injury [157].

4.4. Atrial fibrillation and miRNAs 107, 22-3p and 146a-5p

Within the last two years, miRNAs in atrial fibrillation (AF) have
received increased attention. While a number of studies suggest a relationship of certain miRNAs with incident or recurrent AF [158,159], a recent study by Rivera-Caravanca et al. was able to show a correlation between miRNAs 107, 22-3p and 146a-5p and a higher risk of major cardiovascular events during a mean follow-up of 7.6 years [160]. While miRNAs 22-3p and 107 correlated positively with risk (HR 1.07 and 3.66, respectively), a negative correlation was found regarding miRNA 146a-5p (HR 0.86) [160]. For these miRNAs, however, data is lacking on whether they are regulated by oxidative stress.

5. Advanced redox biomarkers

5.1. sNox2-dp as a read-out of NOX2 activation in human cardiovascular/metabolic disease

Although NADPH oxidases are already investigated for therapeutic targeting since more than a decade, there are still no NOX inhibitors in clinical use for the therapy of CVD [161,162]. So far, there are several promising drug candidates for NOX inhibition in clinical phase II and III trials, although not directly for the therapy of CVD but associated comorbidities [27,163].

The molecular proof to demonstrate the direct association of soluble nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) 2-derived peptide (sNox2-dp) with NOX-2 activity was conducted in patients with chronic granulomatous disease displaying better endothelial function (measured by flow-mediated dilation, FMD) and lower sNox2-dp and 8-iso-PGF2α levels [164]. Mechanistically, a burst of ROS was detected in collagen-stimulated platelets and in phorbol ester-stimulated human neutrophils, which was accompanied by a maximum of sNox2-dp release and could be blocked by matrix metallo proteinase-2 inhibition or the NOX-2 inhibitor Nox2ds-tat [165]. The presence of two comorbidities in children, hypercholesterolemia and obesity, resulted in an additive aggravation of 8-isoprostanes and sNox2-dp as well as impairment of endothelial function (FMD) [166].

Higher sNox2-dp as well as oxidized LDL levels were also observed in children with only one risk factor, hypercholesterolemia [167]. Patients with peripheral artery disease had enhanced sNOX2-dp and isoprostane levels but lower NO-derived metabolites and impaired endothelial function (measured by flow-mediated dilation), all of which correlated with rather low reactivity in the presence of chloride compared to the more reactive hypochlorite (OCl-) in patients with metabolic syndrome [168]. Non-alcoholic steatohepatitis (NASH) showed an association with higher levels of 8-isoprostanes and sNox2-dp, which also showed a correlation with histological grading of steatosis and liver inflammation, ballooning and fibrosis [169]. Non-alcoholic fatty liver disease (NAFLD) was associated with higher sNox2-dp and 8-isoprostane levels in patients, also correlating with more severe steatosis and portal inflammation [170] or with markers of infection [171]. Likewise, peripheral endothelial dysfunction (FMD) in patients with metabolic syndrome significantly diminished the serum sNOX2-dp and urinary 8-iso-PGF2α levels and improved the antioxidant status [173].

Patients with COPD and AF showed higher levels of sNox2-dp and 8-isoprostane, which were additively increased in the presence of both risk factors [174]. Patients with obstructive sleep apnoea syndrome (OSAS) had higher urinary 8-iso-PGF2α and serum sNox2-dp levels but lower NO-derived metabolites and impaired endothelial function (measured by flow-mediated dilation), all of which was ameliorated by continuous positive airway pressure therapy (CPAP) [175].

5.2. Advanced glycation end products

Advanced glycation end products (AGEs) are a group of highly reactive compounds that are formed by reaction of lipids and proteins with AGE precursors such as (methyl)glyoxal [180]. AGE precursors are generated in the presence of high glucose/fructose levels under oxidative stress conditions as observed in diabetic patients but may also accumulate during the aging process [181] and by dietary uptake [182]. Glycation of lipids and proteins can change the activity and biological properties of these biomolecules but AGEs can also promote crosslinking and further oxidation of biomolecules. AGEs can also bind to their receptor (RAGE) and thereby induce inflammation and oxidative stress [183]. In an elderly population of 2111 U.S. subjects free of CVD at baseline, increased levels of carboxymethyl-lysine, a major AGE, were associated with elevated risk of incident cardiovascular events (comprising stroke and/or CAD) after multivariable adjustment (HR 1.11, 95% CI 1.03–1.19 per 1-SD increase) [184]. Likewise, Semba et al. demonstrated increased levels of carboxymethyl-lysine to be associated with increased risk of all-cause (HR 1.84, 95% CI 1.30–2.60 for highest vs. lowest two tertiles) and CVD mortality (HR 2.11, 95% CI 1.27–3.49) in a cohort of 1013 elderly Italian subjects after multivariable adjustment [185]. These observations were also confirmed for subjects without diabetes mellitus. Of note, there are pharmaceutical inhibitors of the AGE/receptor of AGE (RAGE) axis that may be translated to the clinical setting [186].

5.3. Myeloperoxidase expression and activity

Myeloperoxidase (MPO) expression and activity were proposed as suitable redox biomarkers describing a toxifying mechanism of H2O2 with rather low reactivity in the presence of chloride compared to the more reactive hypochlorite (OCl-) [43,161]. In 1090 patients with acute coronary syndrome, MPO levels were assessed to determine its association with death and MI at 6-month follow-up [187]. The results displayed that elevated MPO levels (>350 μg/L) predicted an increased risk of cardiovascular events (HR 2.25, 95% CI 1.32–3.82), which were also shown to extend the prognostic value derived from traditional biochemical markers. In good agreement with these previous observations, MPO levels predicted increased odds of a major adverse cardiac event at 6-month follow-up in 490 subjects with acute chest pain [188]. Likewise, the activity of MPO was previously measured by 3-chlorotyrosine (or 3-bromotyrosine) concentrations in patients with MI by HPLC with electrochemical detection [189], as well as 2-chloroethidium using liquid chromatography with mass spectrometry quantification [190]. Although the clinical evidence for prognostic value of MPO in CVD is so far moderate, this redox biomarker was proposed as a reliable candidate for future routine use (reviewed in Refs. [161,191]). The importance of MPO as a redox biomarker is supported by several pharmacological MPO inhibitors that are currently investigated within phase II trials [27].

5.4. Redox profiling

In principle any protein involved in ROS/RNS generation or degradation can be used as a redox biomarker and measurement of its expression or activity will provide limited mechanistic insights and will
probably also correlate with disease severity or cardiovascular risk. As the number of publications on this topic is huge and even discussion of selected examples would exceed the frame of the present review, we will only add some general considerations here. The expression and activity of antioxidant enzymes such as SODs, catalase, glutathione peroxidases or heme oxygenase-1 represent controversial redox biomarkers as they may be upregulated in one disease and down-regulated in the other. In principle, most antioxidant enzymes are under the control of redox-regulated transcription factors implying that under oxidative stress conditions their expression should be upregulated. However, also expression-suppressing mechanisms may come into play at the epigenetic level or by proteasomal degradation as well as activity of these antioxidant enzymes may be oxidatively inhibited as in the case of Mn-SOD [92]. Indeed, SODs, catalase, glutathione peroxidases are often found down-regulated or inactivated under disease conditions serving as a potential explanation of the observed oxidative stress condition. In contrast, heme oxygenase-1 is often upregulated due to the nature of its regulation via NRF2/KEAP1 that are activated by electrophiles such as ROS, although other regulatory mechanisms exist as well [26]. NRF2/KEAP1 and their antioxidant downstream targets are therefore suitable redox biomarkers that characterize part of the antioxidant defense system and the process of redox homeostasis. Also the activity of dysfunctional redox-sensitive enzymes, e.g. in mitochondria, can be used as a redox biomarker read-out: oxidative inactivation of mitochondrial aldehyde dehydrogenase (ALDH-2) correlated with larger infarct size in mouse models of MI [192] and aconitase activity was associated with lipid oxidation in the human failing heart [193], although both enzymatic activities are far not regularly used as redox biomarkers in the clinical setting.

In addition, proteasomal activity may represent an attractive readout of redox dysregulation as the proteasomal system is subject to redox regulation [194] and changes in proteasomal activity will ultimately lead to the accumulation of oxidatively modified proteins [195]. Therefore, the determination of markers of proteasomal activity by measurement of the concentrations of typical oxidized targets for proteasomal degradation (e.g. protein carbonyls) may provide additional mechanistic insights into dysregulated redox processes. Also impairment of the circadian rhythm is a well-accepted cardiovascular risk factor [196]. Oxidative stress and adverse redox signalling play a central role for the control of the circadian rhythm (also termed ‘redox control of cellular timekeeping’) [197]. A detailed description of harmful redox modifications within the circadian clock system and the impact of environmental stressors therein can be found in Ref. [198].

Also thiol-based enzymes play an important role for antioxidant defense and cellular redox signaling. Thioredoxins, peroxiredoxins and glutaredoxins play an important role for hydrogen peroxide and organic peroxide degradation but also their expression or activity shows no consistent regulatory readout (up- or down-regulation) for different CVD [199]. Therefore, assessment of a larger number of these redox markers, e.g. by (redox) proteomics [200,201] or transcriptomics, will provide more meaningful insights. The resulting “redox profiles” may show a characteristic pattern of up- and down-regulated antioxidant proteins or mRNA for a specific disease condition. A general description of redox analytical approaches can be found in Refs. [202,203], for redox proteomics of S-glutathionylated proteins in Ref. [204], for H2S-modified proteome (known as the sulfhydrome) in Ref. [205] and for a systems biology approach in Ref. [206].

Redox profiling can be also based on assessment of a metabolic pattern. Determination of the GSH/GSSG ratio is the simplest readout of the cellular or serum/plasma redox state, however, with only limited prognostic implications [43]. Assessment of the trans-sulfuration pathway may be a more meaningful approach in the future [207]. Redox lipidomics (or oxidative phospholipidomics) [208,209] and phosphoproteomics [210] represent smaller subsets of the metabolome and proteome that are optimized to assess altered redox processes or oxidative stress conditions. In addition, the content of extracellular vesicles can provide important information on dysregulated redox processes and represent a useful prognostic tool to assess cardiovascular risk [211].

Finally, redox regulation of epigenetic profiles is a well-known process in the cancer field [212,213]. Epigenetic pathways involve a plethora of redox switches such as direct DNA modification by ROS (e.g. 5-hydroxy-methylcytosine or 8-OHdG), redox control of DNA methyltransferases (DNMTs) or DNA demethylases (e.g. TET) via oxygen/H2O2 or redox-sensitive transcription factors, redox control of histone methyl/acyteltransferases (e.g. SMYD1) as well as demethylases/aceetylases (e.g. Junmonji, sirtuins) [37]. The known redox changes of enzymes involved in epigenetic control in heart disease were also previously summarized in full detail [214]. The above mentioned redox regulation of miRNAs and ncRNAs expression by various mechanisms also adds to this concept [39,136]. In general, the genome stability is redox regulated by various epigenetic and classical pathways [38]. The classical genome integrity maintaining mechanisms comprise the redox control of DNA repair mechanisms, mRNA stability (via GAPDH, AP-1 and HIF-1) and control of gene expression by redox-sensitive transcription factors such as NRF2, HIF-1α, OxyR and NFkB. Of note, epigenetic changes are
implicated in the pathogenesis of CVD [215,216]. In the future, the complete characterization of epigenetic profiles in heart disease may allow conclusions on the contribution of redox signaling pathways or specific ROS/RNS to these epigenetic changes and thereby disease severity/progression.

6. Additivity of redox biomarkers in the presence of multiple comorbidities

According to the proposed concept in Fig. 1, patients with different comorbidities should have additive accumulation of redox biomarkers. As a proof of concept, we here present selected examples. Patients with COPD and AF had elevated sNOx2-dp and 8-isoprostane levels that showed an additive increase in the presence of both risk factors (Fig. 4) [174]. 3-NT levels in both abnormal glucose tolerance status and presence of CAD increased compared with the combination of normal glucose tolerance status and the presence of CAD [98]. Smoking, diabetes and body mass index were highly associated with systemic oxidative stress as determined by creatinine-indexed urinary 8-epi-PGF2α levels and a history of CVD [217]. Urinary 8-epi-PGF2α concentrations (normalized to creatinine) were also indicative of the presence of insulin resistance and obesity, as envisaged by a dramatic increase in insulin-resistant patients with a body mass index > 30 [218]. There was also an additive accumulation of 8-iso-PGF2α levels in the urine of CAD patients with multiple comorbidities such as obesity, diabetes mellitus, hypercholesterolemia, hypertension and smoking (Fig. 4) [77]. Levels of the AGE marker, carboxymethyl-lysine, in a population-based study were higher in the presence of the comorbidity diabetes and this additive increase was also associated with higher all-cause and CVD mortality of non-diabetic subjects [185]. Plasma levels of 8-iso-PGF2α gradually increased in individuals that were healthy, underwent coronary angiography without coronary artery atherosclerotic lesions or with proven CAD (123.3 ± 9.5, 314.6 ± 40 and 389.6 ± 36.2 pg/ml, respectively) [219]. Importantly, in the patient group, there was a substantial increase in 8-iso-PGF2α concentration in the presence of hypertension as compared to the normotensive group (394.2 ± 42.7 and 232.7 ± 25.1 pg/ml, respectively) and also patients with dyslipidemia displayed higher 8-iso-PGF2α levels (359.1 ± 35.6 and 240.3 ± 34.3 pg/ml). Finally, this study revealed a positive correlation between 8-iso-PGF2α concentrations and age. At the functional level, the presence of the typical cardiovascular comorbidities smoking and hypercholesterolemia aggravated endothelial dysfunction (measured by impaired acetylcholine-dependent forearm blood flow increases by plethysmography) in an additive manner [220]. This trend was also mirrored by the plasma levels of autoantibodies against oxidized LDL, which was significantly elevated in smokers with hypercholesterolemia.

In a study with 46 patients with rheumatoid arthritis endothelial function, as assessed by FMD and coronary flow reserve, and nitro-oxidative stress, as assessed by 3-NT and MDA, were shown to be related to left ventricular myocardial deformation in rheumatoid arthritis patients [221]. In a double-blind crossover trial in 80 patients with rheumatoid arthritis (60 with CAD and 20 without) patients with CAD had 3-fold higher protein carbonyl, 3-NT, and MDA levels compared to the ones without CAD [222]. In a study of 100 poorly-controlled diabetic patients the reduction of oxidative stress was associated with improved arterial elasticity and myocardial deformation after 12 months of intensive antidiabetic treatment indicating that a decrease of oxidative stress burden was associated with a greater improvement of vascular function and myocardial deformation [223]. In 115 newly diagnosed untreated patients with primary systemic amyloidosis, increased FMD was found to be an independent predictor of all-cause mortality. Increased 3-NT levels were observed in high-risk amyloidosis patients with elevated FMD and hypotension, which may be secondary to increased inflammation and INOS activity, as exhaled NO gas as well as 3-NT levels were also higher in these patients [224]. A major cardiovascular comorbidity is the aging process and increased (mitochondrial) oxidative stress, impaired endothelial function as well as higher cardiovascular risk in the elderly is well documented [225]. The accumulating oxidative damage with increasing age is also supported by exacerbated lipofuscin concentrations in the lysosomal system of the elderly, where lipofuscin represents a highly aggregated mixture of cross-linked proteins, peroxidized lipids and carbohydrates [226]. Of note, lipofuscin itselfs represent a source of ROS and trigger apoptosis, inhibit the proteasomal system and cause mitochondrial dysfunction [227], all of which leads to dysregulated proteostasis and thereby to a loss of a functional proteome due to insufficient removal of damaged/inactive proteins [226]. Despite this appealing concept, at present there are no large scale clinical studies or meta-analyses that link the aging process with accumulation of lipofuscin, whereas accumulation of lipofuscin in the retina seems to be associated with age-related macular degeneration [228]. However, support for an association of oxidative stress and aging comes from a study of 2190 subjects undergoing a health care examination by showing substantial relations to total antioxidant status, total peroxides and autoantibodies against oxidized LDL with age and body mass index [229]. These results were confirmed in smaller studies that demonstrated aging to be correlated with increased oxidative stress or impaired antioxidant defense [230–236]. Moreover, the MARK-AGE project revealed rather moderate associations of markers of oxidative stress or antioxidants with progressing age [237]. Only 3-NT showed a weak correlation with age and higher levels against the middle-aged control group, whereas no comparable trend was found for protein carbonyls and MDA. Only the antioxidant lycopene was lower in all higher age groups, whereas reduced cysteine levels even tended to increase with higher age. The authors concluded that lifestyle and environmental factors have a more pronounced impact on age-related redox biomarkers than previously anticipated [237]. Whereas the levels of most redox biomarkers seem to correlate with the progression or severity of a certain disease, so far there are no clear threshold values that could be used for early disease diagnosis, especially since most cardiovascular disease display a multi-comorbidity profile. Therefore, better redox biomarkers or even profiles are needed for a reliable cardiovascular risk stratification and the establishment of redox biomarkers in clinical routine use.

7. Conclusions and outlook

There are basically two major classes of redox biomarkers (Fig. 2). The classical markers of oxidative stress can neither provide specificity for the nature of ROS/RNS nor for certain disease/comorbidities but they have prognostic implications (Fig. 3 and Table 1) and show additivity in the presence of different disease/comorbidities (Fig. 4). The more meaningful redox profiling techniques (e.g. redox proteomics, lipidomics and epigenomics) may help in the future to add the missing specificity to redox diagnostics, to understand and better discriminate beneficial and harmful ROS/RNS processes (e.g. dysregulated kinase, proteasomal, circadian networks) and to identify highly specific redox targets for the development of novel redox drugs. The most promising non-classical redox biomarkers, even when measured as a single parameter, could be NOX enzymes, NFR2 and MPO as they are also subject therapeutic targeting within advanced clinical phase II-III trials on multiple disease categories [26,27]. Combining of these redox biomarkers with functional measurements, e.g. redox-sensitive endothelial function using flow-mediated dilation or acetylcholine-dependent increases in peripheral blood flow by plethysmography [9,238], may further improve their prognostic value. Endothelial function itself also correlates with markers of oxidative stress and redox biomarkers [239–241]. In addition, direct measurement of ROS/RNS formation using specific probes can provide valuable information on the nature, source and disease mechanisms but are hardly applicable to large clinical trials as storage of samples will usually modify the readout due to autooxidation of the probes or inactivation of enzymatic sources in a time-dependent manner. Other protocols for assessment of redox-related...
biomarkers or biological changes with relevance for preclinical and clinical studies on cardioprotection (e.g. oxygen consumption or mitochondrial membrane potential) were previously summarized in a compendium of practical guidelines [242]. Fig. 5 summarizes the prognostic value of the classical markers of oxidative stress and features of other redox biomarkers. Further support for the prognostic value of redox biomarkers (mostly oxidative stress parameters) comes from multiple studies on pharmacological antioxidant interventions leading to a normalization of these redox biomarkers as well as functional parameters (reviewed in Ref. [18]).

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**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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