Oxidative status in different settings and with different methodological approaches compared by Receiver Operating Characteristic curve analysis

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Abstract

Objectives: To test the performance of different analytical approaches in highlighting the occurrence of deregulated redox status in various physio-pathological situations.

Design and Methods: 35 light and 61 heavy smokers, 19 chronic renal failure, 59 kidney transplanted patients, and 87 healthy controls were retrospectively considered for the study. Serum oxidative stress and antioxidant status, assessed by spectrophotometric Reactive Oxygen Metabolites (d-ROMs) and Total Antioxidant Capacity (TAC) tests, respectively, were compared with plasma free (F-MDA) and total (T-MDA) malondialdehyde, both quantified by isotope-dilution gas chromatography-mass spectrometry (ID-GC-MS). Sensitivity, specificity and cut-off points of T-MDA, F-MDA, d-ROMs and TAC were evaluated by both receiver-operator characteristic (ROC) analyses and area under the ROC curve (AUC).

Results: Only T-MDA assay showed clearly absence of oxidative stress in controls and significant increase in all patients (AUC 1.00, sensitivity and specificity 100%). Accuracy was good for d-ROMs (AUC 0.87, sensitivity 72.8%, specificity 100%) and F-MDA (AUC 0.82, sensitivity 74.7%, specificity 83.9%), but not high enough for TAC to show in patients impaired antioxidant defence (AUC 0.66, sensitivity 52.0%, specificity 92.9%).

Conclusions: This study reveals T-MDA as the best marker to detect oxidative stress, shows the ability of d-ROMs to identify modified oxidative status particularly in the presence of high damages, and evidences the poor TAC performance. D-ROMs and TAC assays could be useful for routine purposes; however, for an accurate clinical data evaluation, their comparison versus a “gold standard method” is required.

Keywords: Antioxidant status; isotope-dilution-gas chromatography-mass spectrometry; malondialdehyde; methods comparison; oxidative stress; ROC curve analysis.

Introduction

Oxidative stress plays a role in different human pathological conditions; it is caused by an imbalance between reactive oxidants (endogenous and exogenous) and antioxidant defense systems [1-5]. Measuring the body's reaction to oxidants' attack is clinically useful to disclose possible oxidative imbalances, depletion of antioxidant defenses, disease progression and response to medical treatment. To this purpose, different biomarkers have been proposed as indexes of lipid peroxidation (hydroperoxides, dienes, F2-isoprostanes, malondialdehyde) and/or various endogenous antioxidant compounds (e.g., thiol groups, Vit A, Vit E, antioxidant enzymes) [6]. Ideally these methods should be used synergistically rather than as homologous competitive oxidative stress biomarkers, however this is unfeasible from the practical point of view, so it is important to distinguish the most suitable one for the diverse indications.

MDA, the most abundant lipid peroxidation end-product, arising from the attack of ROS on PUFAs with at least 3 double bonds, is present both in a free form (F-MDA) chemically active and a potentially damaging agent, and bound (B-MDA) to nucleophilic groups (SH or NH_2) of proteins and lipoproteins, indicative of an older injury and excreted by urine [7, 8]. Usually MDA is determined by reaction with thiobarbituric acid as thiobarbituric acid-reactive substances (TBARS), an assay not totally specific because other aldehydes rather than MDA can be quantified [1, 3, 5-7, 9]. Moreover, the standard TBARs procedure measures only total MDA (T-MDA), i.e. the sum of F-MDA and B-MDA, while for F-MDA evaluation, a proteins precipitation step is needed before the derivatization reaction. Nonetheless, a new assay has been developed which exhibits high specificity and sensitivity both for free and protein bound MDA [10].

According to the European Food Safety Authority (EFSA), MDA could be used as an index of oxidative damage if appropriate techniques are available for its quantification [11].

The high metrological level method based on gas chromatography-mass spectrometry with isotope dilution (ID-GC-MS) [12] allows both the direct measurement of F- and T-MDA concentrations, avoiding proteins precipitation. The indirect measurement of B-MDA is calculated as the difference between the total and free MDA amounts. The use of dideuterated MDA as internal standard suppresses bias occurring during hydrolysis, derivatization, extraction, and ensures the accuracy of the analytical process by adequate calibration curves. The attempt to search for an alternative non deuterated internal standard seems also hopeful [13, 14].

Despite this highly sensitive and specific technique is expensive, time-consuming and affordable by a limited number of specialized laboratories, it may be conveniently used to confirm the results obtained by other simpler methods, more rugged and workable in a clinical routine.

Some commercial assays are available either to provide a global picture of several potentially harmful oxidant compounds (deriving from a large class of biological molecules after the radicals' attack) or to assess the serum/plasma barrier to oxidation, as total antioxidant capacity (TAC) [3, 15-22]. These assays allow to measure oxidative status in a quicker and easier way and are suitable for routine applications in a clinical laboratory setting.

In our previous paper on critically ill patients in intensive care units [23], the sensitivity of the commercial assay (d-ROMs, Reactive Oxygen Metabolites), which determines hydroperoxides, an acute intermediate product arising from the attack of ROS on PUFAs [24, 25], was reported to be lower than MDA determination by ID-GC-MS [12]. The poor performance of dROMs test [23] was attributed to many factors such as the small number of patients enrolled in the study, their highly critical conditions and the complex concurrent pharmacological treatment. The unsatisfactory performance of d-ROMs was in agreement with the reports of some authors [16, 19], but in marked contrast to the findings of other groups, who introduced the same assay for clinical routine measurements [15, 17, 18, 20-22, 24-27].

Here we present a retrospective study on light and heavy smokers (LS, HS), chronic renal failure patients (CRF) and kidney transplant patients (KP), in order to ascertain the suitability of F-MDA, B-MDA, T-MDA, d-ROMs and TAC tests in different clinical settings characterized by oxidative stress. Thus, results from previous clinical studies [26, 28-30], have been collected and statistically compared for the first time, with the aim to search for the best marker and/or assay and to verify their performance in evidencing oxidative stress.

Materials and Methods

Subjects

The subjects analyzed in this study had been previously enrolled for different research projects approved by the local Institutional Ethics Committee and carried out according to the guidelines of the Helsinki Declaration. Written informed consent was obtained from all the subjects fulfilling the inclusion criteria.

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a) LS and HS groups. Light smokers (LS) (≤ 12 cigarettes per day for at least 2 years) and heavy smokers (HS) (≥ 20 cigarettes/day for at least 10 years), aged between 30-60 years, were recruited from a pool of volunteers (Department of Pneumology, Ospedale Niguarda; Clinica del Lavoro "Devoto", Ospedale Maggiore Policlinico, Milan, Italy). They were in good health as indicated by a general medical questionnaire and by chest X-ray, pulmonary function, absence of respiratory complications by spirometry, electrocardiogram and clinical laboratory parameters. Exclusion criteria: presence of chronic diseases, cardiovascular episodes within 6 months (coronary artery disease, peripheral or cerebral vascular disease), regular medication, hypertension (defined as systolic or diastolic blood pressure higher than 140 or 100 mmHg, respectively), impaired renal function, body mass index (BMI <19 or >25 kg/m^2), hyperglycemia, mellitus diabetes. The healthy controls were all non-smokers (n=57, M/F 30/27, aged 42.3±11.5 yrs) [28, 30].

b) CRF group. Chronic renal failure patients (CRF), enrolled at the Nephrology Unit (Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy), were on a conservative dietary and drug treatment to control their clinical conditions. All the patients, in the 20-80 years range, were selected on the basis of a renal clearance value <20 mL/min in two separate instances. Exclusion criteria: acute infection or peritonitis during the two months before the study started, neoplasm, severe malnutrition and severe hypoalbuminemia (<3 g/dL), liver cirrhosis, clinically symptomatic cardiac or vascular diseases, heavy smoking (>20 cigarettes/day for a year before recruitment). The study included also healthy controls (n=30, M/F 18/12, aged 59±9) [29].

c) KT Group. Eligibility criteria for patients who received a kidney transplant (KT) from cadaver or living donors at the Nephrology Unit (Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy): at least 6 months follow-up with a stable plasma creatinine ≤2.5 mg/dL. The immunosuppressive treatment consisted in cyclosporine A plus prednisone with or without azathioprine-mycophenolate mofetil, tacrolimus plus prednisone with or without azathioprine-mycophenolate mofetil. Exclusion criteria: patients with a double organ transplant, patients who had had any type of previous cardiovascular diseases (cerebral ischemia, atrial fibrillation, angina) and patients with ~~mellitus diabetes~~ diabetes mellitus at the time of transplant [26]. The concentrations of all MDA forms in KT patients were never published before. No healthy controls were enrolled in this study.

Samples collection

Peripheral venous blood samples were drawn after overnight fasting; 2 blood specimens from each patient were collected in light protected tubes, either with no additive for serum d-ROMs and total antioxidant capacity (TAC) measurement or containing ethylenediaminethetraacetic acid (EDTA) to prevent coagulation. EDTA specimens were immediately centrifuged to obtain plasma samples for MDA determination. All samples were frozen and stored at –80 °C until biochemically measured.

Analytical methods

Serum reactive oxygen metabolites were measured by a spectrophotometric method using the d-ROMs test commercial kit on FREE analyzer (Diacron International, Grosseto, Italy), as previously described [17, 28]. The test evaluates the ability of *in vivo* formed hydroperoxides to generate *in vitro* alkoxy and peroxyl radicals in the presence of transition metals acting as catalyzers. When free radicals react with a correctly buffered chromogenic substance, they develop a coloured complex showing the higher absorbance at 505 nm. The concentration of the coloured complex is directly proportional to the concentration of hydroperoxides. The resulting d-ROMs values were obtained in arbitrary units (U.Carr., Carratelli Units), then converted into mmol/L of H_2O_2, as 1 CARR U is stated by the manufacturer to be equivalent to 0.08 mg/dL. The sensitivity of the d-ROMS test was 0.26 mM H_2O_2, and the method was linear up to 267 mmol/L. Intra- and inter-assay CV's were 2.07 and 1.79%, respectively

Serum TAC was measured by a spectrophotometric method using the OXY-Adsorbent test commercial kit on FREE analyzer (Diacron International, Grosseto, Italy), as previously described [26]. The test is based on the ability of hypochlorous acid (HClO) to oxidize the physiologic antioxidants (uric acid, glutathione, thiol groups, vitamins, glutathione peroxidase, superoxide dismutase and catalase). As HClO reacts with a correctly buffered chromogenic substrate, it forms a coloured complex that can be measured photometrically at 505 or 546 nm. The concentration of the coloured complex is directly proportional to the concentration of HClO and indirectly proportional to the antioxidant ability of the sample. The analytical imprecision of the test is: CV within-run = 1.90%; CV between-run = 2.05%.

Plasma free and total MDA were measured following the procedures described by Cighetti et al. [12]. For free MDA, plasma (0.2 mL) was diluted with citric buffer (0.4 mol/L; pH 4.0), added to butylated hydroxytoluene (0.5 mmol/L; 5 nmol) and synthesized dideuterated MDA (0.25 nmol) as internal standard. Samples, derivatized with phenylhydrazine (50 mmol/L; 1 µmol) for 30 min at room temperature, were extracted with hexane and analysed by GC-MS.

For total MDA, plasma (0.2 mL), added with dideuterated MDA (0.25 nmol) as internal standard, was hydrolyzed in 1 mol/L NaOH at 60 °C for 60 minutes, then treated as described for free MDA. Bound MDA fraction was calculated as the difference between total and free MDA. The addition of butylated hydroxytoluene to the plasma samples as antioxidant and the low temperature for hydrolysis and derivatization reactions avoid the formation of interfering compounds. Moreover, the use of dideuterated MDA added to the biological samples as internal standard before any pre-analytical step, together with the availability of synthesized, purified and crystallized MDA, allows the measurement of true free and total MDA values using adequately prepared calibration curves [12]. The analytical imprecision of the assay is: CV within-run and between-run = 1.0 and 1.2% for F-MDA, 0.9 and 1.1% for T-MDA, respectively. The laboratory cut-off for free- and total MDA is 0.50 and 2.10 µmol/L, respectively, derived from the mean +2SD of 500 determinations in healthy subjects enrolled over 10 years in different clinical studies (data not shown).

Statistical analyses and Methods comparison

The performance of the assays, indicating the ability to discriminate between subjects with increased and those with unmodified oxidative status, was evaluated by the Receiver Operating Characteristic (ROC) curve analysis [31] providing the corresponding cut-off values with the highest accuracy (minimal false negative and false positive results). The cut-off values of each assay were identified as those maximizing the ϕ correlation coefficient $(TP \cdot TN - FP \cdot FN) / \sqrt{((TP + FN) \cdot (TN + FP) \cdot (TP + FP) \cdot (TN + FN))}$, where TP = true positive, FP = false positive, TN = true negative and FN = false negative, sqrt = square root [32, 33]. The cut-off thresholds were then derived and the area under the ROC curve (AUC), sensitivity (recognizing increased oxidative stress when it is truly present, i.e. identifying true positive rate: TP / (TP + FN)) and specificity (recognizing the true absence of oxidative stress, i.e. identifying true negative rate: TN / (TN + FP)) were calculated, along with their 95% confidence intervals (CI), estimated via bootstrap (1000 runs). The assays were evaluated by comparing their AUC and by reporting the statistical significance of the difference between the areas under different ROC curves. All analyses were performed with the R System [34]. The strength of association between variables, for data not normally distributed, was identified by the robust pairwise Spearman's coefficient and the corresponding 95% Confidence Intervals (CI). Post-hoc power analysis has been performed using the pwr libraries [35]. The power analysis and the significance were set at 0.8 and $p < 0.05$, respectively.

Results

Groups whose oxidative stress parameters were retrospectively compared in this study include: all the apparently healthy volunteers enrolled in the different clinical studies as controls (CTR) (n=87; M/F 48/39; aged 47.7±10.7 ys), light smokers (LS) (n=35; M/F 25/10; aged 50.0±7.0), heavy smokers (HS) (n=61; M/F 35/26; aged 51.0±10.1), chronic renal failure patients (CRF) (n=19; M/F 14/5; aged 60.0±16.0) and kidney transplant patients (KT) (n=59; M/F 34/25; aged 52.5±8.4).

The trend for each measured parameter among the groups is presented in Figure 1. Only T-MDA could discriminate between the absence of oxidative stress in controls and the presence of oxidative damage in all the four studied groups. F-MDA was the only marker not significantly increased in CRF patients compared to controls (only 32% of subjects had abnormal F-MDA), while the other two MDA forms (total and bound), d-ROMs and TAC were significantly altered.

As regards LS subjects, 92% and 71% showed F- and B-MDA, respectively, above their cut-off values, while only 34% had d-ROMs in pathologic ranges. TAC value was low for each group compared to controls, indicating a considerably altered oxidative status as confirmed by all the other assays, except 64% of KT patients who maintained a good antioxidant capacity.

The excellent T- MDA assay performance was confirmed by the highest accuracy for both sensitivity and specificity (100%) according to ROC curve analyses cut-off (T-MDA <2.10 µmol/L) and with the highest AUC value (= 1.000), indicating complete separation between true positive and true negative values. Compared to the highest T-MDA accuracy (Table 1), F-MDA presented almost the same ability to distinguish between true positive (74.7% sensitivity) and true negative values (83.9% specificity), whereas B-MDA slightly deferred from T-MDA only for sensitivity (94.3%). The d-ROMs test recognized the true negative (100% specificity) much better than the true positive values (72.8% sensitivity). TAC assay showed the lowest sensitivity identifying only 52% of the true positive values and the lowest AUC indicating a small difference in distribution between true positive and true negative.

The strength of correlations among the markers obtained by pairwise Spearman test is reported in Table 2. T-MDA showed a significant correlation with all the variables examined except with TAC. Interestingly T-MDA seems stronger related with d-ROMs than with F-MDA. F-MDA is the only MDA form which correlates with TAC (negative weak correlation). dROMs and TAC do not correlate.

Discussion

Several methods exists for monitoring oxidative status in physio-pathological, nutritional or living conditions, however the poor performance of the commercial assays and their inadequacy to distinguish between healthy controls and patients is still under debate, and results obtained by different analytical methods are often difficult to compare [19, ~~35~~ 36]. Nevertheless, the use of commercial kits tested in the present study is still widespread among clinical and research laboratories for their simplicity of use and the low cost, so an additional study on their performance in different clinical settings merits attention.

In this retrospective study, we evaluated the performance of two commercial assays, d-ROMs and TAC, which measure "families" of parameters involved in oxidative stress, to study the unbalance of oxidative status and antioxidant barrier caused by smoking habit and/or various diseases. Their ability to provide correct diagnoses was compared with free and total MDA assessed by ID-GC-MS [12], a high metrological level method which measures a single and chemically defined parameters (the endogenously formed MDA). Of course the results were not compared in term of magnitude since the measured indexes are chemically different, but only in terms of diagnostic potency. To do this the widely accepted ROC curve analysis [~~36-38~~ 37-39] was used instead of other statistical procedures such as Passing and Bablok-regression or Passing and Bablok-residuals.

Of all the measured parameters (Figure 1), only T-MDA confirmed the lack of oxidative stress in healthy controls and the presence of oxidative damage in all the four studied groups with different severity from light smokers to CRF patients. The T-MDA excellent performance was confirmed by the highest accuracy for both sensitivity and specificity (100%) as evidenced by ROC curve analyses. Consequently, we compared all the methods for the assessment of oxidative status against T-MDA, considered as our reference index (gold standard).

Very different information was provided by the other assays. As concerns as F-MDA, it was unable to show CRF groups increased oxidative status (Figure 1). However, its outcome in CRF group is not totally surprising as F-MDA, much lower than T-MDA (less than 10%), is highly reactive and may be expected to be variable and not cumulative. Thus, the biochemical meaning of the different MDA forms needs further speculation to explain for the significant, but not strong correlations, between F-MDA and both B- and T-MDA (Table 2). The endogenous F-MDA levels depend on the balance between MDA formation and its detoxification as B-MDA, cleared by kidneys in urine after its transformation from protein-MDA adducts to simpler MDA-adducts by proteolytic enzymes [8]. Thus, increased T-MDA

is not only indicative of increased lipid peroxidation in all pathological conditions, but can also arise from reduced kidney functionality, as in chronic renal insufficiency and in dialysis patients [29], leading to the B-MDA values in CRF higher than other groups (Figure 1). Similarly, unchanged F-MDA concentration is not only indicative of the lack of lipid peroxidation, but also represents the MDA amount remaining after F-MDA conjugation with proteins and aminoacids [7, 8]. Therefore, all the three MDA forms must be considered to frame the true oxidative status of a sample [29].

Concerning the d-ROMs test accuracy, its specificity was close to T-MDA in identifying true negative values, while its sensitivity was weaker (Table 1). This assay was able to reveal increased oxidative stress in a high percentage of CRF, KT and heavy smokers, but failed to identify most of the true positive light smoker subjects, as instead determined by the MDA forms (Figure 1). Thus, the d-ROMs sensitivity could rely on the intensity of the oxygen radical formation. In fact, the smoking damage originated by many hazardous products reacting as electrophilic compounds, causes an oxidative stress condition related to long lasting smoking and to the number of cigarettes/day; hence, heavy smokers should have a higher grade of oxidative stress than light smokers. The different performance of d-ROMs observed in this study suggests that this test is unable to assess small hydroperoxide amounts *in vivo* formed and further *in vivo* transformed into F-MDA, as in LS, whereas it is suitable to measure intense and continuous hydroperoxides formation, as in HS, CRF and KT.

Therefore, d-ROMs assay could be partially influenced by the prevalence of the disease (increased oxidative stress due to high hydroperoxide amounts well identified in pathological conditions) [~~39~~ 40], or by the pool of endogenous antioxidants and/or diet supplementations or drugs, modifying the indirect measurement of the *in vivo* formed hydroperoxides that are *in vitro* transformed into alkoxy and peroxyl radicals by the assay procedure. This statement could explain the ability of the d-ROMs test to show either a modified oxidative status as reported by some authors [15, 18, 20-22], or the lack of its performance as reported by others [1, 16, 19, 23]. Notably, the d-ROMs test showed weak but significant correlations with the MDA forms evaluated by ID-GC-MS (Table 2), although these assays are based on totally different principles.

Poor performance was observed for TAC assay showing the lowest sensitivity and AUC area (Table 1). Nevertheless, in all the groups except KT patients, this assay evidenced a disrupted serum antioxidant barrier (Figure 1), as a consequence of endogenous antioxidants consumption to counteract the formation and/or attack of free radicals. The preserved total antioxidant steady-state in KT patients, despite a pro-oxidant environment as evidenced by all

the other tests, suggests the presence of either a basal stronger protection than in other groups, or some analytical interference due to co-administered pharmacological therapies. Thus, the evaluation of antioxidant ability alone might not be sufficient to identify the change in oxidative status but needs to be accompanied by other assays [16 ~~40~~].

Study limitations: a) Comparison between MDA levels by ID-GC-MS and those resulting from the HPLC-TBAs assay has not been included. b) In the clinical studies considered for the present retrospective analysis [26, 28, 29, 30], the evaluation of plasma Vitamin E was never carried out as the changing of oxidative status was detected by total and reduced glutathione, cysteine, homocysteine and cysteinylglycine. On the other hand, when Vitamin E was measured [37], in contrast to significant increase in F-MDA and reduction in GSH, no significant changes of both alfa- and gamma tocopherol (the two Vit E isoforms) were observed in patients *vs* controls.

In conclusion, the results of this retrospective study show that T-MDA, quantified by ID-GC-MS method, is, among the evaluated markers or assays, the best index for evidencing oxidative stress. Our study also confirms the ability of the d-ROMs test to show modified serum oxidative status particularly in the presence of high damages and evidences the poor sensitivity of TAC assay. These two commercial assays are easy to perform, suitable for screening tests and useful for routine clinical purposes. Moreover, they give the possibility of assessing simultaneously the balance/unbalance of oxidative status. In case of critical clinical conditions, a more reliable and sensitive assay should be mandatory. Validation of routine against the "gold standard method" could help biochemists to acquire accurate data for intra-laboratory standardization and clinicians to evaluate data more correctly.

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