

Technical Note

Improved integrative analysis of the thiol redox proteome using filter-aided sample preparation

Elena Bonzon-Kulichenko^{a,b,1}, Emilio Camafeita^{a,b,*,1}, Juan Antonio López^{a,b,1},
 María Gómez-Serrano^{b,c,2}, Inmaculada Jorge^{a,b}, Enrique Calvo^{a,b}, Estefanía Núñez^{a,b},
 Marco Trevisan-Herraz^{a,b,3}, Navratan Bagwan^{a,4}, José Antonio Bárcena^{d,e}, Belén Peral^c,
 Jesús Vázquez^{a,b}

^a Cardiovascular Proteomics Laboratory, Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid, Spain

^b Centro de Investigación Biomédica en Red de Enfermedades Cardiovasculares (CIBERCV), Madrid, Spain

^c Instituto de Investigaciones Biomédicas, Alberto Sols, (IIBM), Consejo Superior de Investigaciones Científicas & Universidad Autónoma de Madrid (CSIC-UAM), Madrid, Spain

^d Dept. Biochemistry and Molecular Biology, University of Córdoba, Córdoba, Spain

^e Maimónides Biomedical Research Institute of Córdoba (IMIBIC), Córdoba, Spain

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ABSTRACT

Changes in the oxidation state of protein Cys residues are involved in cell signalling and play a key role in a variety of pathophysiological states. We had previously developed GELSILOX, an in-gel method that enables the large-scale, parallel analysis of dynamic alterations to the redox state of Cys sites and protein abundance changes. Here we present FASILOX, a further development of the GELSILOX approach featuring: i) significantly increased peptide recovery, ii) enhanced sensitivity for the detection of Cys oxidative alterations, and iii) streamlined workflow that results in shortened assay duration. In mitochondria isolated from the adipose tissue of obese, diabetic patients, FASILOX revealed a sexually dimorphic trait of Cys oxidation involving mainly mitochondrial oxidative phosphorylation complexes. These results provide the first evidence for a decreased efficiency in the antioxidant response of men as compared to women.

While the generation of reactive nitrogen and oxygen species (RNOS) has a favourable impact on a number of signal transduction pathways involving reversible reduction and oxidation of specific amino acids [1], their accumulation is known to be behind life-threatening dysfunctions like cardiovascular disease [2–4], cancer [5,6] and neurological disorders [7,8]. Hence, measuring redox damage is central to deciphering disease pathogenesis. Among many other chemically feasible oxidative posttranslational modifications (PTMs) that may occur in proteins, redox reactions involving Cys thiol groups are widespread, frequently reversible and enzymatically controlled [9,10]. However, the lability and low abundance of Cys oxoforms have hampered the large-scale characterization of the specific Cys residues that

sense the oxidative milieu, which for decades could only be tackled by site-directed mutagenesis [11].

A number of proteomics approaches to protein Cys thiol oxidation have been proposed for the enrichment, detection and quantitation of such modifications (reviewed in [12]). These quantitative, MS-based methods have not only unveiled a variety of redox-regulated thiol proteins, but also pinpointed the precise reactive Cys residues and quantitated their oxidation level. However, their reliance on isolating and enrichment of Cys-containing peptides prevents parallel evaluation of protein abundance variations in a single experiment. In 2012 we introduced the in-gel method GELSILOX (GEL-based Stable Isotope Labelling of OXidized Cys), which took advantage of the

* Corresponding author at: Cardiovascular Proteomics Laboratory, Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid, Spain.

E-mail address: ecamafeita@cnic.es (E. Camafeita).

¹ In alphabetical order; these three authors contributed equally to this work.

² Present address: Dr. Gómez-Serrano's present address is Institute of Molecular Biology and Tumor Research (IMT), Center for Tumor Biology and Immunology (ZTI), Philipps University, Marburg, Germany.

³ Present address: Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom.

⁴ Present address: Department of Biomedical Sciences, Affiliated with The Novo Nordisk Foundation Center for Protein Research, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark.

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polyacrylamide matrix used for SDS-PAGE as a protein reaction chamber to perform the sequential steps of alkylation and reduction needed to separately label reduced and oxidized Cys sites without sample losses [13]. GELSILOX combined the differential alkylation of Cys peptides with stable isotope labelling (SIL) and *ad-hoc* statistical analysis based on the WSPP (weighted spectrum, peptide and protein) model [14] and became the only approach capable to provide simultaneous quantitation of proteins and reversible Cys oxoforms [13]. Since then, GELSILOX has revealed alterations to the thiol redoxome originating from different pathological processes in several species, including mouse and rat heart cardiomyocytes after the ischemia-reperfusion insult [13,15,16], aged mouse cardiomyocytes [17], mouse fibroblasts upon reoxygenation [18], bovine aortic endothelial cells exposed to laminar shear stress [19] and adipocytes isolated from human obese individuals [20]. In the meanwhile, filter-aided sample preparation (FASP) [21] has become the method of choice for protein digestion, as it combines detergent-based universal protein solubilisation with efficient proteome clean-up before digestion, while circumventing the limitations of the gel format, notably low peptide recovery. In FASP, the sample is solubilized in 4% SDS and retained in an ultrafiltration device that acts as a support for detergent removal, buffer exchange and protein digestion.

In view of the advantages of FASP over in-gel (and over in-solution) procedures, in this work we sought to investigate whether the ultrafiltration device could be used as a reaction chamber to host the

sequential reactions needed for differential alkylation of Cys sites. We also analyzed peptide recovery, sensitivity against Cys oxoforms, assay duration and quantitative accuracy of the filter-based method. The resulting procedure, which we called FASILOX (Filter-Aided Stable Isotope Labelling of OXidized Cys) consists of the following steps (Fig. 1): a) protein extraction in the presence of SDS and a first alkylating agent (e.g. iodoacetamide, IAM); b) protein loading onto filter and SDS removal; c) reduction of reversibly oxidized thiol groups with a reducing agent (e.g., DTT); d) washing and treatment with a second alkylating agent (e.g. MMTS); e) washing, tryptic digestion of proteins and elution of peptides; f) differential labelling of peptides *via* SIL; g) LC-MS/MS analysis; h) statistical analysis with the WSPP model using SanXoT [22] for simultaneous quantification of the proteome and the Cys redoxome. Note that additional steps of treatment and washing may be added when necessary.

Comparative performance of FASILOX and GELSILOX. The performance of FASILOX was benchmarked against that of GELSILOX using mouse embryonic fibroblasts (MEFs) treated with PBS (control) or subjected to chemical oxidation with 2 mM diamide (treated) for 10 min (four biological replicates per condition, see Section 2 of Supplementary Methods for details). The so-obtained eight cell homogenates were split in two aliquots for comparative FASILOX and GELSILOX analysis. Before SIL, 3- μ g aliquots from the digests of two control and two treated samples were analyzed by LC-MS/MS using a 90-min gradient (Supplementary Methods, Section 4). As shown in Fig. 2 A-D,

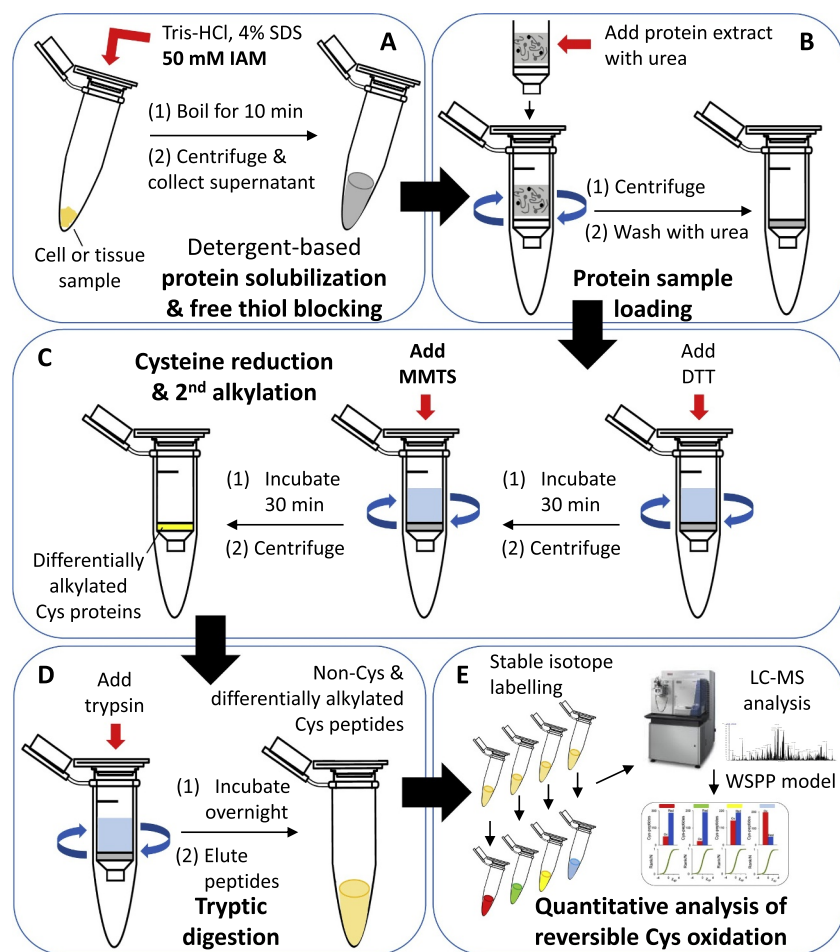


Fig. 1. The FASILOX workflow. (A) A cell or tissue homogenate sample is boiled in the presence of SDS (for protein release and denaturation) and IAM (to alkylate free thiol, natively reduced groups), after which the sample is centrifuged and the supernatant collected. (B) The protein extract is diluted in urea and loaded onto the filter device, which is then washed with urea for SDS removal. (C) Reversibly oxidized protein thiol groups are reduced with DTT and then alkylated using the second alkylating agent, MMTS. (D) On-filter tryptic digestion of proteins results in a mixture of non-Cys and differentially alkylated Cys peptides. (E) Peptides are differentially tagged *via* SIL and analyzed by LC-MS/MS. Statistical analysis based on the WSPP model enables parallel quantitation of dynamic alterations to the redox state of Cys sites and protein abundance changes. IAM, iodoacetamide; MMTS, *S*-methyl methanethiosulfonate; SIL, stable isotope labelling; WSPP, weighted spectrum, peptide and protein.

FASIOX outperformed GELSILOX not only in terms of the total number of peptide-spectrum matches (PSMs) identified (12,389 vs. 7696; Fig. 2 A; see Section 5.1 of Supplementary Methods), but also, interestingly, in the number of Cys PSMs (2201 vs. 1485; Fig. 2 C). In terms of unique peptides, FASIOX yielded 7234 total unique peptides on average considering both control and treated samples, and 216 oxidized Cys peptides with the diamide-treated samples. This represents 41% and 78% increase with respect to the amounts reached by GELSILOX (5115 and 121, respectively; Supplementary Table 1).

The two 8-digest sets (GELSILOX and FASIOX), obtained from the four control and four treated samples, were independently labeled with iTRAQ (Supplementary Methods, Section 2.3), joined and subjected to LC-MS/MS analysis using a long (6 h) gradient (Supplementary Methods, Section 4) followed by statistical analysis based on the WSPP model [14] using the software package SanXoT [22] (Supplementary Methods, Section 5.2). FASIOX allowed the quantitation of 8953 peptides, out of which 1221 (14%) were Cys-containing peptides, whereas GELSILOX yielded 5757 peptides (36% less than FASIOX) including 847 (15%) Cys-containing peptides (31% less than FASIOX) (Fig. 2 D and Supplementary Table 2). A total of 2979 unique non-Cys peptides and 498 unique Cys-containing peptides were quantitated by both approaches, which represents ca. 60% of the unique peptides found with GELSILOX (Fig. 2 E and F and Supplementary Table 2). The quantitative values of the peptides prepared using GELSILOX followed a strict normal distribution in all the cases (Fig. 2 G, black curves), as expected [13]. Notably, the peptides prepared using FASIOX also followed the expected normal distribution in the four replicates (Fig. 2 G, black curves), indicating that their error distribution could also be accurately modeled using the WSPP model. In addition, and as expected, diamide treatment increased the abundance of many oxidized Cys-containing peptides (Fig. 2 G, red curves), with concomitant decrease in the abundance of reduced Cys-containing peptides (Fig. 2 G, blue curves), being the effect equally evident for both GELSILOX and FASIOX methods. As expected for a treatment that triggers Cys oxidation rather than altering protein levels, most of the peptides showing significant abundance changes upon diamide treatment, as revealed by the standardized variable at the peptide level, z_{pq} , turned out to be Cys-containing peptides (Supplementary Table 2). Of note, the pattern of significant changes in Cys-containing peptides, is very similar between FASIOX and GELSILOX (Fig. 2 H); however, FASIOX showed 65% increased sensitivity for the detection of such abundance changes (Supplementary Table 2).

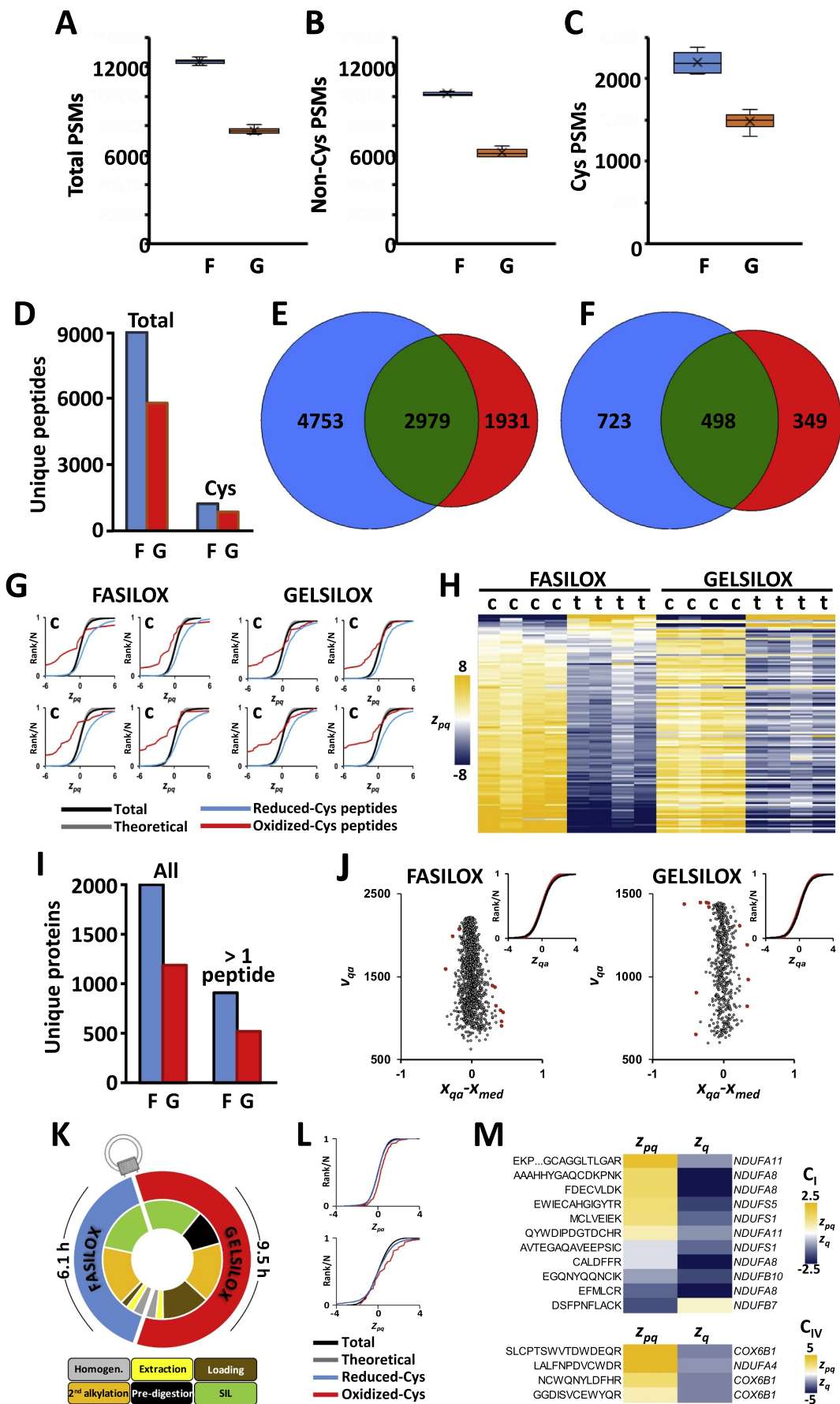
Considering non-Cys peptides, 1992 and 907 unique proteins were quantified in the MEF cells based on FASIOX and GELSILOX, respectively (1184 and 520 when considering only proteins identified with at least two peptides, Fig. 2 I and Supplementary Table 3). As was the case with peptide quantitation, the quantitative protein values followed a strict normal distribution by both FASIOX and GELSILOX, showing that the WSPP model described very accurately the protein variability associated to both procedures (Fig. 2 J). Consistently, less than ten significant protein abundance changes were found at 5% FDR (Fig. 2 J). This finding confirmed the fact that in the MEF cells diamide treatment resulted in significant alterations of Cys oxidation levels with negligible effect on protein abundances. Altogether, these results demonstrate that FASIOX enables accurate protein quantitation with parallel assessment of oxidative alterations in Cys-containing peptides, largely outperforming GELSILOX in terms of peptide recovery, sensitivity against Cys oxidative alterations and assay duration (Fig. 2 K).

Application of FASIOX to study the role of gender in the mitochondrial subproteome of obese, diabetic patients. Next, we tested FASIOX on visceral adipose tissue samples to tackle the role of gender in the oxidative damage caused by obesity and type 2 diabetes mellitus (T2DM) in human patients. For that, mitochondria isolated from visceral adipocytes of male obese patients diagnosed with T2DM

were compared to those from their female counterparts based on an iTRAQ SIL experiment (see Sections 3 and 4 of Supplementary Methods). A total of 15,762 unique peptides pertaining to 2895 unique proteins were identified; of these, 2399 (15%) turned out to be Cys-containing peptides, most of them (83%) reduced (Table 1 and Supplementary Table 4). Of note, the proteins identified (Supplementary Methods, Section 5.1) include 742 of the 1114 (67%) human genes with mitochondrial localization according to the Human MitoCarta2.0 inventory [23] (Supplementary Table 4). The number of mitochondrial PSMs was very similar to that of non-mitochondrial PSMs (Table 1); taking into account that the mitochondrial content of adipocytes is estimated to be around 10% [24], this means that the mitochondrial content was enriched ca. five times.

The statistical analysis using the WSPP model (Supplementary Methods, Section 5.2) revealed that the male samples contained increased levels of oxidized Cys-containing peptides, as revealed by the standardized variable at the peptide level (without correction for protein mean) z_{pa} (Fig. 2 L). When the analysis was circumscribed to the 754 MitoCarta mitochondrial proteins, z_{pq} , the standardized variable at the peptide level that takes into account the protein mean, showed that regardless of protein abundance changes, the male group of obese, diabetic patients had increased abundance of Cys oxidations in mitochondrial proteins (Fig. 2 L). It is noteworthy that 17 out of the 64 Cys-oxidized mitochondrial peptides for which z_{pq} could be calculated belong to oxidative phosphorylation (OXPHOS) complexes, notably complexes I and IV, which showed diminished abundance in men as compared to women (Fig. 2 M and Supplementary Table 5). In particular, three of the peptides that were found the most strongly oxidized in men belong to *COX6B1* and one to *NDUFA4*, both of them members of OXPHOS complex IV that in turn showed strongly decreased abundance in men (Fig. 2 M and Supplementary Table 5). Remarkably, upregulation of the same Cys-containing peptides concomitant with strong downregulation of *COX6B1* and *NDUFA4* has been recently reported for T2DM patients in a redox proteomics study with obese women [20], where complex IV was pointed out as target for oxidative modifications linking aging and T2DM. Moreover, a distinctive male and female phenotype regarding antioxidant response had been suggested in a proteomics study with T2DM, obese patients based on the differential abundance of proteins like superoxide dismutase, glutathione S-transferase and glutathione peroxidase, among others [25]. The results attained in this work using FASIOX not only substantiate this sexually dimorphic phenomenon, but also highlight a less efficient antioxidant response in men as compared to women.

In summary, we demonstrate here that an ultrafiltration membrane (FASIOX) may be used as reaction chamber instead of a polyacrylamide matrix (GELSILOX) for performing thiol redox proteomics, clearly increasing performance in terms of peptide recovery and sensitivity against Cys oxidative modifications. In addition, increased simplicity of FASIOX results in shortened assay duration and facilitates assay automation. FASIOX can easily incorporate additional treatments that require intermediate washing steps. This is the case of chemistries that are able to distinguish among S-nitrosylation, S-glutathionylation and S-acylation [26], which would benefit from the negligible sample loss of FASIOX without requiring very large amounts of protein. FASIOX is also compatible with isotopically-labeled thiol reagents, which would label reduced and reversibly-oxidized Cys sites with the same chemistry. These reagents allow estimation of the fraction of oxoforms at each Cys site [27], although, due to isotopomer overlapping, they are not compatible with the use of additional isotope labels for relative protein quantitation. In mitochondria isolated from the adipose tissue of obese, diabetic patients, the high peptide recovery and sensitivity against Cys oxoforms characteristic of FASIOX have provided the first evidence for a decreased efficiency in the antioxidant response of men as compared to women.



(caption on next page)

Fig. 2. FASIOX results. (A-C) Comparative FASIOX (blue) and GELSILOX (red) analysis on the same MEF samples. The graphs show the number of total (A), non-Cys (B) and Cys (C) PSMs. (D) Total and Cys-containing unique peptides quantified in the MEF samples after SIL using FASIOX (blue) and GELSILOX (red). (E) Distribution of the total unique peptides quantitated by FASIOX (blue) and GELSILOX (red) with overlapping peptides shown in green. (F) Distribution of the Cys-containing unique peptides quantitated by FASIOX (blue) and GELSILOX (red) with overlapping peptides shown in green. (G) Cumulative distribution of z_{pq} , the standardized value at the peptide level, for the total (black), reduced-Cys (blue) and oxidized-Cys (red) peptides quantitated in the four control MEF samples with FASIOX and GELSILOX. The theoretical distribution $N(0,1)$ is also drawn (grey). Positive/negative z_{pq} values indicate increased/decreased peptide abundance with respect to the average of the four replicate diamide-treated samples. (H) Significant abundance changes ($FDR_{pq} < 0.01$) in Cys-containing peptides across MEF samples by FASIOX and GELSILOX in terms of z_{pq} values. Only peptides quantitated by both FASIOX and GELSILOX were included. Positive/negative z_{pq} values indicate increased/decreased peptide abundance with respect to the average of the four replicate samples corresponding to the opposite treatment and are shown in orange/blue, respectively. (I) Number of total unique proteins and proteins identified with more than one peptide that were quantified in the MEF samples after SIL using FASIOX (blue) and GELSILOX (red). (J) Weighted distribution of log-ratio protein quantitation around the experiment grand mean using FASIOX (Left) and GELSILOX (Right). Red dots indicate significant protein abundance changes found at 5% FDR. The inset shows the corresponding cumulative distribution of z_{qa} , the standardized value at the protein level, neglecting the contribution of Cys-containing peptides (black curve; the theoretical normal distribution is shown in red). (K) Comparison of the time required to carry out the different steps of FASIOX and GELSILOX procedures: cell or tissue homogenization (grey); preparation of the protein extract (yellow); sample loading (comprising proteome concentration in the stacking/resolving gel interface followed by protein staining with Coomassie blue and water/acetonitrile washes in the case of GELSILOX, brown); incubation with second alkylation agent (comprising prior protein reduction with DTT, orange); pre-digestion (consisting on gel permeation with cold trypsin in the case of GELSILOX, black); and SIL of tryptic peptides (green). Overnight tryptic digestion, common to both methods, and overnight SDS-PAGE gel polymerization, unique to GELSILOX, have been omitted for simplification. (L) Cumulative distribution of z_{pa} (Top) and z_{pq} (Bottom), standardized values at the peptide level without and with correction for protein mean, respectively, for the total (black), reduced-Cys (blue) and oxidized-Cys (red) peptides quantitated in the men vs. women comparative of obese, diabetic patients using FASIOX. The theoretical distribution $N(0,1)$ is included (grey). Positive/negative z_{pa} or z_{pq} values indicate increased/decreased peptide abundance in men as compared to women. (M) Abundance changes in OXPHOS complex I (Top) and complex IV (Bottom) oxidized-Cys peptides (in terms of z_{pa}) and their corresponding complex protein components (in terms of z_q) in the men vs. women comparative of obese, diabetic patients using FASIOX. Positive/negative z_{pa} or z_q values indicate increased/decreased peptide or protein abundance in men as compared to women. Abbreviations: c, control sample; C_i, OXPHOS complex I; C_{IV}, OXPHOS complex IV; F, FASIOX; G, GELSILOX; OXPHOS, oxidative phosphorylation; PSM, peptide-spectrum match; t, treated sample. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

PSMs, peptides and proteins identified in adipocyte mitochondria from obese, diabetic patients.

	PSMs	Total / Cys Peptides	Proteins
Total	96,575	15,762 / 2399	2895
Non-mitochondrial	48,687	9206 / 1382	2141
Mitochondrial	47,888	6556 / 1017	754

Cys: Cys-containing; PSM: peptide-spectrum match.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jpropt.2019.103624>.

Declaration of Competing Interest

The authors declare no conflict of interest.

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References

- [1] K.M. Holmstrom, T. Finkel, Cellular mechanisms and physiological consequences of redox-dependent signalling, *Nat. Rev. Mol. Cell Biol.* 15 (2014) 411–421.
- [2] V. Kumar, T. Kleffmann, M.B. Hampton, M.B. Cannell, C.C. Winterbourn, Redox proteomics of thiol proteins in mouse heart during ischemia/reperfusion using ICAT reagents and mass spectrometry, *Free Radic. Biol. Med.* 58 (2013) 109–117.
- [3] A.P. Halestrap, S.J. Clarke, I. Khaliulin, The role of mitochondria in protection of the heart by preconditioning, *Biochim. Biophys. Acta* 1767 (2007) 1007–1031.
- [4] D.M. Yellon, D.J. Hausenloy, Myocardial reperfusion injury, *N. Engl. J. Med.* 357 (2007) 1121–1135.
- [5] F. Weinberg, N.S. Chandel, Reactive oxygen species-dependent signaling regulates cancer, *Cell. Mol. Life Sci.* 66 (2009) 3663–3673.
- [6] D.C. Wallace, Mitochondria and cancer, *Nat. Rev. Cancer* 12 (2012) 685–698.
- [7] D.A. Butterfield, A. Grjec, H.F. Poon, A. Castegna, W.M. Pierce, J.B. Klein, R.N. Martins, Redox proteomics identification of oxidatively modified brain proteins in inherited Alzheimer's disease: an initial assessment, *J. Alzheimers Dis.* 10 (2006) 391–397.
- [8] M.T. Lin, M.F. Beal, Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases, *Nature*. 443 (2006) 787–795.
- [9] M. Lindahl, A. Mata-Cabana, T. Kieselbach, The disulfide proteome and other reactive cysteine proteomes: analysis and functional significance, *Antioxid. Redox Signal.* 14 (2011) 2581–2642.
- [10] J.M. Held, B.W. Gibson, Regulatory control or oxidative damage? Proteomic approaches to interrogate the role of cysteine oxidation status in biological processes, *Mol. Cell. Proteomics* 11 (2012) R111 013037.
- [11] Y.M. Janssen-Heininger, B.T. Mossman, N.H. Heintz, H.J. Forman, B. Kalyanaraman, T. Finkel, J.S. Stamler, S.G. Rhee, A. van der Vliet, Redox-based regulation of signal transduction: principles, pitfalls and promises, *Free Radic. Biol. Med.* 45 (2008) 1–17.
- [12] K. Wojdyla, A. Rogowska-Wrzęsinska, Differential alkylation-based redox proteomics—lessons learnt, *Redox Biol.* 6 (2015) 240–252.
- [13] P. Martinez-Acedo, E. Nunez, F.J. Gomez, M. Moreno, E. Ramos, A. Izquierdo-Alvarez, E. Miro-Casas, R. Mesa, P. Rodriguez, A. Martinez-Ruiz, D.G. Dorado, S. Lamas, Vazquez J. a novel strategy for global analysis of the dynamic thiol redox proteome, *Mol. Cell. Proteomics* 11 (2012) 800–813.
- [14] P. Navarro, M. Trevisan-Herraz, E. Bonzon-Kulichenko, E. Nunez, P. Martinez-Acedo, D. Perez-Hernandez, I. Jorge, R. Mesa, E. Calvo, M. Carrascal, M.L. Hernaez, F. Garcia, J.A. Barcena, K. Ashman, J. Abian, C. Gil, J.M. Redondo, J. Vazquez, General statistical framework for quantitative proteomics by stable isotope labeling, *J. Proteome Res.* 13 (2014) 1234–1247.
- [15] M. Ruiz-Meana, E. Nunez, E. Miro-Casas, P. Martinez-Acedo, I. Barba, A. Rodriguez-Sinovas, J. Inserte, C. Fernandez-Sanz, V. Hernando, J. Vazquez, D. Garcia-Dorado, Ischemic preconditioning protects cardiomyocyte mitochondria through mechanisms independent of cytosol, *J. Mol. Cell. Cardiol.* 68 (2014) 79–88.
- [16] C. Fernandez-Sanz, M. Ruiz-Meana, J. Castellano, E. Miro-Casas, E. Nunez, J. Inserte, J. Vazquez, D. Garcia-Dorado, Altered FoF1 ATP synthase and susceptibility to mitochondrial permeability transition pore during ischaemia and reperfusion in aging cardiomyocytes, *Thromb. Haemost.* 113 (2015) 441–451.
- [17] C. Fernandez-Sanz, M. Ruiz-Meana, E. Miro-Casas, E. Nunez, J. Castellano, M. Loureiro, I. Barba, M. Poncelas, A. Rodriguez-Sinovas, J. Vazquez, D. Garcia-Dorado, Defective sarcoplasmic reticulum-mitochondria calcium exchange in aged mouse myocardium, *Cell Death Dis.* 5 (2014) e1573.
- [18] A. Guaras, E. Perales-Clemente, E. Calvo, R. Acin-Perez, M. Loureiro-Lopez, C. Pujol, I. Martinez-Carrascoso, E. Nunez, F. Garcia-Marques, M.A. Rodriguez-Hernandez, A. Cortes, F. Diaz, A. Perez-Martos, C.T. Moraes, P. Fernandez-Silva, A. Trifunovic, P. Navas, J. Vazquez, J.A. Enriquez, The CoQH2/CoQ ratio serves as a sensor of respiratory chain efficiency, *Cell Rep.* 15 (2016) 197–209.
- [19] F.J. Sanchez-Gomez, E. Calvo, R. Breton-Romero, M. Fierro-Fernandez, N. Anilkumar, A.M. Shah, K. Schroder, R.P. Brandes, J. Vazquez, S. Lamas, NOX4-dependent hydrogen peroxide promotes shear stress-induced SHP2 sulfenylation and eNOS activation, *Free Radic. Biol. Med.* 89 (2015) 419–430.
- [20] M. Gomez-Serrano, E. Camafeita, J.A. Lopez, M.A. Rubio, I. Breton, I. Garcia-Consuegra, E. Garcia-Santos, J. Lago, A. Sanchez-Pernaute, A. Torres, J. Vazquez, B. Peral, Differential proteomic and oxidative profiles unveil dysfunctional protein import to

- adipocyte mitochondria in obesity-associated aging and diabetes, *Redox Biol.* 11 (2017) 415–428.
- [21] J.R. Wisniewski, A. Zougman, N. Nagaraj, M. Mann, Universal sample preparation method for proteome analysis, *Nat. Methods* 6 (2009) 359–362.
- [22] M. Trevisan-Herraz, N. Bagwan, F. Garcia-Marques, J.M. Rodriguez, I. Jorge, I. Ezkurdia, E. Bonzon-Kulichenko, J. Vazquez, SanXoT: a modular and versatile package for the quantitative analysis of high-throughput proteomics experiments, *Bioinformatics.* 35 (9) (2018) 1594–1596.
- [23] S.E. Calvo, K.R. Clauser, V.K. Mootha, MitoCarta2.0: an updated inventory of mammalian mitochondrial proteins, *Nucleic Acids Res.* 44 (2016) D1251–D1257.
- [24] S. Boudina, T.E. Graham, Mitochondrial function/dysfunction in white adipose tissue, *Exp. Physiol.* 99 (2014) 1168–1178.
- [25] M. Gomez-Serrano, E. Camafeita, E. Garcia-Santos, J.A. Lopez, M.A. Rubio, A. Sanchez-Pernaute, A. Torres, J. Vazquez, B. Peral, Proteome-wide alterations on adipose tissue from obese patients as age-, diabetes- and gender-specific hallmarks, *Sci. Rep.* 6 (2016) 25756.
- [26] N.S. Gould, P. Evans, P. Martinez-Acedo, S.M. Marino, V.N. Gladyshev, K.S. Carroll, H. Ischiropoulos, Site-specific proteomic mapping identifies selectively modified regulatory cysteine residues in functionally distinct protein networks, *Chem. Biol.* 22 (2015) 965–975.
- [27] P. Liu, Y.Q. Huang, W.J. Cai, B.F. Yuan, Y.Q. Feng, Profiling of thiol-containing compounds by stable isotope labeling double precursor ion scan mass spectrometry, *Anal. Chem.* 86 (2014) 9765–9773.