RESEARCH ARTICLE

Oxidative stress occurs early in Down syndrome pregnancy: A redox proteomics analysis of amniotic fluid

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Purpose: The present study aims to evaluate a set of oxidative stress biomarkers in the amniotic fluid (AF) of women carrying Down syndrome (DS) fetuses that could prove in vivo the early occurrence of oxidative damage in DS.

Experimental design: To assess the extent of protein oxidation in DS AF, we measured protein carbonylation and protein-bound HNE by slot-blot analysis, total and oxidized GSH levels by enzymatic assay and heat shock proteins (HSPs) thioredoxin (Trx) induction by Western blot. Further, by a redox proteomics approach specific targets of protein carbonylation were identified.

Results: We found increased levels of oxidative stress, as indexed by increased protein oxidation, lipid peroxidation, reduction of GSH and Trx levels and induction of the HSP response. By a redox proteomics approach, we identified selective proteins which showed increased oxidation in DS fetuses compared with healthy controls. The identified proteins are involved in iron homeostasis (ceruloplasmin and transferin), lipid metabolism (zinc-α2-glycoprotein, retinol-binding protein 4 and apolipoprotein A1) and inflammation (complement C9, α-1B-glycoprotein, collagen α-1V chain) with critical relevance in the clinical outcome of DS.

Conclusions and clinical relevance: Our results indicate that oxidative damage is an early event in the DS pathogenesis and might contribute to the development of deleterious DS phenotypes, including abnormal development and AD-like neuropathology.

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Abbreviations: AF, amniotic fluid; **ApoA1,** apolipoprotein A1; **CP,** ceruloplasmin; **DNPH,** 2,4-dinitrophenylidrazine; **DS,** Down syndrome; **DTNB,** 5,5′-dithiobis 2-nitrobenzoic acid; **IGFBP-1,** insulin-like growth factor-binding protein 1; **IP,** immunoprecipitation; **OS,** oxidative stress; **RBP4,** retinol-binding protein 4; **SOD,** superoxide dismutase; **Trx,** thioredoxin; **ZAG,** zinc-α-2-glycoprotein

1 Introduction

Down syndrome (DS) is a chromosomal abnormality characterized by reduced cognitive ability and Alzheimer-like dementia, several features of the individual's phenotype and accelerated ageing [1]. DS is better described as a multifactorial disease characterized by an abnormal expression of trisomic genes arising not only from genetic but also environmental factors [2]. Several studies demonstrated a major role of oxidative stress (OS) in DS clinical phenotypes [3–6]. The main and most investigated mechanism that has been proposed to explain the enhanced OS in DS is the

increased intracellular activity of cytosolic copper/zinc superoxide dismutase (SOD-1). It has been proposed that many of the neurological symptoms of DS result from the increased activity of SOD, even though there is still an open debate [7]. The increase in SOD activity, not accompanied by a similar increase in the expression levels of catalase and glutathione peroxidase, can cause an imbalance in steady-state oxidative status, owing to the formation of hydrogen peroxide. Once formed, this compound can react with reduced form of redox-active transition metal ions, producing the dangerous oxidant, hydroxyl radical [8] that causes oxidative damage to biological macromolecules. Considering that oxidative modifications of proteins normally lead to their dysfunction, such changes can be deleterious to the cell and can represent one of the principal targets of OS-induced modification resulting in cellular impairment.

A significant increase in oxidative DNA damage (urinary 8-OHdG), lipid peroxidation and isoprostane 8,12-iso-iPF2α-VI levels [3, 5, 9–12] was reported in urine obtained from DS children and DS amniotic fluid (AF) samples, respectively, indicating a ''pro-oxidant state'' which, associated with overwhelmed antioxidant defences, contribute to shed light on the complexity of the DS phenotype. Indeed, an abnormal expression of genes located on chromosome 21, in association with responses to environmental stimuli, might also regulate the expression of disomic genes [1].

Few studies investigated the role of OS in the early stage of DS pathogenesis and the majority have been performed on DS fetal brains. Mitochondrial dysfunction has been reported to occur already during embryonic development [13]. However, there is lack of data on AF, which is a more reliable index of the physiological condition of the fetus. The biochemical composition of AF, routinely used for prenatal diagnosis, is modified throughout pregnancy and its protein profile reflects both physiological and pathological changes affecting the fetus and the mother [14].

In the present study, we sought to elucidate the involvement of OS in the pathogenesis of DS phenotypes through the evaluation of multiple OS parameteres. In addition, by a redox proteomics approach we identified the specific oxidatively modified proteins. The roles of the identified proteins with their relevance to DS pathogenesis are also discussed.

2 Materials and methods

2.1 Sample collection

A retrospective matched case control study was performed in collaboration with Artemisia Fetal-Maternal Medical Centre, Rome, Italy. AF samples (20 mL) were obtained from women undergoing amniocentesis in the 15–17th week of gestation. AF was collected from ten women carrying normal fetuses and ten samples from women with positive diagnosis of DS. All AF samples did not contain any blood contamination as indexed by red blood cell and hemoglobin concentration (data not shown). Following centrifugation for the collection of amniocytes for cytogenetic analysis, supernatants were centrifuged again at $12\,000 \times g$ at 4°C for 30 min for the removal of insoluble components. All samples used as controls were obtained from women who had normal uneventful deliveries at term. Written informed consent was obtained from all subjects to donate AF for research purposes. The study was approved by the Ethics Committee of the Artemisia Medical Group (Rome, Italy).

2.2 Sample preparation

AF (5 mL) was mixed with acetone 1:3 v/v and kept overnight at −20°C. After centrifugation at $12\,000 \times g$ for 10 min, the supernatant was discarded and the pellet was resuspended in 0.5 mL of Media 1 (0.32 M sucrose, 0.1 mM $MgCl_2$, 0.1 mM EDTA. 10 mM Tris-HCl, pH 8.0) containing 1 mM PMSF and 1 mg/mL aprotin. Samples were let in ice for 30 min and then sonicated (10 s three times) and then centrifuged at $18\,000 \times g$ for 10 min. Protein concentration was measured in the supernatant using the Coomassie Plus Pierce Protein Assay (Rockford, IL, USA). Sample aliquots (300 μg protein) were then depleted for albumin and IgG by using the ProteoPrep Blue Albumin Depletion kit (Sigma-Aldrich, Milan, Italy) following the manufacturer's instructions. After depletion, protein concentration was measured again as described above.

2.3 Protein oxidation measurement

Protein oxidation was measured according to Butterfield et al. [15]. Briefly, samples (5 μL) were added with 5 μL of 12% SDS and derivatized with 10 mM 2,4-dinitrophenylidrazine (DNPH) at room temperature for 20 min. Samples were neutralized with 7.5 μL of neutralization solution (2 M Tris in 30% glycerol). Derivatized samples (250 ng) were then blotted onto a nitrocellulose membrane under vacuum using a slot-blot apparatus (Bio-Rad). Membranes were blocked with 3% BSA in TBS-T for 1 h and next incubated with rabbit antibody to protein-bound DNP (diluted 1:150) for 90 min. After washing with TBS-T, membranes were incubated with anti-rabbit IgG alkaline phosphatase secondary antibody (1:5000) in TBS-T for 1 h at room temperature. The membrane was washed in TBS-T and developed using a solution of NBT (0.2 mM) and BCIP (0.4 mM) in alkaline phosphate buffer (0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$; pH 9.5). Dried blots were quantified using QuantityOne image analysis (Bio-Rad).

Protein-bound HNE were detected using the above-described process strategy without derivatization. The membranes were incubated with anti-protein-bound HNE

rabbit polyclonal antibody (1:5000) for 2 h, washed with TBS-T and then incubated with an anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody (1:10 000). Blots were developed with BCIP/NBT SigmaFast tablets (Sigma), dried and quantified as described above.

2.4 Reduced and oxidized glutathione assay

Reduced and oxidized glutathione contents were measured by the NADPH-dependent GSSG reductase method as described by Calabrese et al. [16]. For total glutathione, an aliquot (0.1 mL) of sample was added to 0.1 mL of a solution containing 5 mM EDTA in 100 mM potassium phosphate, pH 7.5 (buffer 1) and 10 mM 5,5′-dithiobis 2-nitrobenzoic acid (DTNB). The samples were then mixed by tilting and centrifuged at $12\,000 \times g$ for 2 min at 4°C. An aliquot (100 μL) of the supernatant was added to a cuvette containing 0.5 U of GSSG reductase (Sigma) in buffer 1. After 1 min of equilibration, the reaction was initiated with 220 nmol of NADPH in buffer 1 for a final reaction volume of 1 mL. The formation of a GSH-DTNB conjugate was then measured at 412 nm in a Cary Varian Spectrophotometer. For assay of GSSG, aliquots (0.25 mL) of samples were added to 0.25 mL of a solution containing 10 mM *N*-ethylmaleimide (NEM) in buffer 1. After mixing and centrifuging the sample as above, supernatant was passed at one drop/s through a SEP-PAK C18 Column (Waters, Framingham, MA, USA) that had been washed with methanol followed by water. The column was then washed with 1 mL of buffer 1. Aliquots of the combined eluates were added to a cuvette with 250 nmol of DTNB and 0.5 U of GSSG reductase. The assay then proceeded as in the measurement of total GSH. The total glutathione of a sample was calculated from a standard curve of GSH concentration versus absorbance.

2.5 Two-dimensional gel electrophoresis

Samples (200 μg of proteins) were incubated at room temperature for 30 min either in four volumes of 10 mM DNPH in 2 N HCl for protein carbonyl derivatization or 2 N HCl for gel maps and mass spectrometry analysis, according to the method of Levine et al. [17]. All the details about the procedure are described in our previous works [18, 19].

For the first-dimension electrophoresis, 200 μL of sample solution were applied to a ReadyStrip™ IPG strip pH 3–10 (Bio-Rad Laboratories, Milan, Italy). The strips were soaked in the sample solution for 1 h to allow uptake of the proteins. The strips were then actively rehydrated in Protean IEF Cell Apparatus (Bio-Rad) for 16 h at 50 V. The isoelectric focusing was performed at 300 V for 2 h linearly; 500 V for 2 h linearly; 1000 V for 2 h linearly, 8000 V for 8 h linearly and 8000 V for 10 h rapidly. All the processes above were carried out at room temperature. The focused IEF strips were stored at −80°C until second dimension electrophoresis was performed.

For second dimension electrophoresis, thawed strips were equilibrated for 10 min in 50 mM Tris-HCl (pH 6.8) containing 6 M urea, 1% w/v SDS, 30% v/v glycerol, and 0.5% DTT, and then re-equilibrated for 15 min in the same buffer containing 4.5% iodacetamide in place of DTT. 12% Precast criterion gels (Bio-Rad) were used to perform second dimension electrophoresis. Precision Protein™ Standards (Bio-Rad) were run along with the sample at 200 V for 65 min.

After electrophoresis, the gels were incubated in fixing solution (7% acetic acid, 10% methanol) for 20 min. Approximately 40 mL of Bio-Safe Coomassie Gel Stain (Bio-Rad) were used to stain the gels for 1 h, on a gently continuous rocker. The gels were placed in deionized water overnight for destaining.

2.6 Western blot

The gels were prepared in the same manner as 2-D-electrophoresis. The proteins from the second dimension electrophoresis gels were transferred to nitrocellulose (Bio-Rad) using Criterion Blotter apparatus (Bio-Rad) at 100 V for 1 h. The DNP adducts of the carbonyls of the proteins were detected on the nitrocellulose paper using a primary rabbit antibody (Millipore, MA, USA) specific for DNP-protein adduct (1:100), followed by a secondary goat anti-rabbit IgG alkaline-phosphatase conjugated antibody (Sigma). The resultant stain was developed using BCIP/NBT SigmaFast tablets (Sigma).

HSP 70, heme oxygenase 1 (HO-1,) Grp 78 and thioredoxin (Trx) were also evaluated by Western blot analyses. In brief, proteins (40 μg) for each sample were separated by a 12.5% SDS-PAGE and electroblotted (1 h at 100 V) to nitrocellulose membranes (Bio-Rad) using 25 mM Tris, 192 mM glycine and 20% v/v methanol. Equal protein loading was confirmed by staining with 0.2% v/v Ponceau S in 7% acetic acid. Blotted membranes were blocked with 5% non-fat dried milk and challenged with appropriate primary antibodies, namely anti-HSP70 mouse monoclonal antibody (1:500) (Stressgen, Ann Arbor, MI, USA), anti HO-1 (1:500) (Abnova GmbH, Heidelberg, Germany), Grp 78 rat monoclonal antibody and anti-Trx rabbit monoclonal antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) for 1 h at room temperature. Unbound antibodies were removed by washing TBS-T for 5 min. The membranes were then incubated with HRP-conjugated secondary antibody diluted 1:5000. Protein bands were visualized with ECL PlusTM (Amersham, NJ, USA) according to the manufacturer's protocol. Blots were scanned on a GS-800 densitometer (Bio-Rad) and quantified by QuantityOne image software.

2.7 Image analysis

The 20 gels ($n = 10$ controls and $n = 10$ DS) and 20 nitrocellulose blots were scanned and saved in TIF format using

a GS-800 densitometer (Bio-Rad). PDQuest 2-D Analysis software (version 7.2.0, Bio-Rad) was used for matching and analysis of visualized protein spots among differential gels and membranes, to compare protein and DNP immunoreactivity content between DS and CTR samples. To identify valid spots, PDQuest spot detection software was used as reported in our previous works [20]. Statistical significance was assessed by a two-tailed Student's *t*-test, the method of statistical analysis most appropriate for proteomic analysis of small number of protein spots [21]. *p*-Values <0.05 were considered significant for comparison between control and experimental data (DS versus CTR).

2.8 Trypsin digestion and protein identification by mass spectrometry

Selected spots was manually excised from gel and submitted to trypsin proteolysis according to procedure already reported by Canettieri et al. [22]. Briefly, after one destaining steps using 50 mM ammonium bicarbonate (15 min), 50% ACN in 50 mM ammonium bicarbonate (10 min) and 100% ACN (15 min), about 100 ng of trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega, Madison, WI, USA), solubilized in 10 μL of a 25 mM ammonium bicarbonate digestion buffer, were added to vacuum-dried gel. Digestion was performed at 37°C overnight. An aliquot of the peptide mixture was mixed with the same volume of CHCA matrix solution (5 mg/mL) in 70% ACN containing 0.1% TFA v/v and spotted onto an appropriate MALDI target plate. MALDI-ToF MS analyses were performed in a Voyager-DE STR instrument (Applied Biosystems, Framingham, MA) equipped with a 337 nm nitrogen laser and operating in reflector mode. Mass data were obtained by accumulating several spectra from laser shots with an accelerating voltage of 20 kV. Two tryptic autolytic peptides were used for the internal calibration (m/z 842.5100 and 2807.3145).

The MS data were analysed by MoverZ program (v. 2002, http://bioinformatics.genomicsolutions.com), according to default parameters. Identification by peptide mass fingerprint (PMF), with the monoisotopic mass list, after exclusion of expected contaminant mass values by Peak Erazor program (http://www.protein.sdu.dk/gpmaw/Help/PeakErazor/peakerazor.html), was performed using the MASCOT search engine (v. 2.2) against human Swiss-Prot database [Swiss-Prot 2010_04 (516 081 sequences; 181 677 051 residues)]. Up to one missed cleavage, 50 ppm measurement tolerance, oxidation at methionine (variable modification) and carbamidomethylation at cysteine (fixed modification) were considered. Identifications were validated when the probability-based Mowse protein score was significant according to MASCOT [23].

2.9 Protein identification by LC-MS/MS

Selected peptide mixtures were separated by on-line reverse-phase (RP) capillary liquid chromatography and analyzed by electrospray tandem mass spectrometry (ESI-MS/MS). The samples were loaded onto a 15 cm reverse-phase fused-silica capillary column (BioBasic-18, inner diameter 180 μm, 300 Å, 5 μm, Thermo Scientific), using Dionex Ultimate 3000 system (LC Packings, Dionex, Amsterdam, The Netherlands), by an autosampler. Peptides were fractionated with a 70 min gradient from 5 to 95% ACN in 0.1% of formic acid at a flow rate of 2 μL/min. The HPLC system was connected to a linear ion trap-orbitrap hybrid mass spectrometer (LTQ-Orbitrap Discovery, Thermo Fisher Scientific, Bremen, Germany) equipped with a nanoelectrospray ion source (Thermo Fisher Scientific), operating in the positive ionization mode with a spray voltage of 1.9 kV. The eluted peptides were detected in a precursor MS scan mode by Orbitrap (300–2000 m/z, 30 000 resolution at m/z 400), followed by sequential data-dependent MS/MS scans in which the three most abundant ions were fragmentated in CID and analyzed in the linear trap (minimal signal required 2000, isolation width of 3 m/z, normalized collision energy 35%, removal of 1+ions or ions with unassigned charge state and selection of 2+, 3+ and 4+ ions).

2.10 Database search

The MS/MS spectra were searched by Sequest algorithm (Proteome Discoverer, v. 1.0, Thermo Fisher Scientific, San Jose, CA) against UniProtKB/Swiss-Prot database (Release 2010_05, 516 603 sequence entries, 181 919 312 residues). Searching parameters were established as fixed modification of Cys (+57.0215 Da, alkylation by iodoacetamide), dynamic modification of oxidized Met (+15.9949 Da), up to two missed cleavages for trypsin digestion, tolerance at 10 ppm and 0.8 Da for precursor ions and fragment ions, respectively. Also considered were a, b and y ion series for database match. The following filter criteria were applied: at least two peptides per protein and XCorr values of 2.0, 2.25 and 2.5 for doubly, triply and quadruply charged precursor ions, respectively.

2.11 Immunoprecipitation (IP)

The IP was performed as described previously [24]. Anti-ApoA1 (apolipoprotein A1) antibody (10 μL) was added directly to AF samples (200 μg of proteins) with IP Buffer (NaCl 0.15 M, NP-40 0.5%, Tris-HCl 50 mM pH 7.2, protease inhibitors), and the mixture was incubated on a rotary mixer overnight at 4°C. The antigen/antibody complex was precipitated by adding 50 μL of protein-A-conjugated agarose beads (0.1 M in PBS), mixed on a rotary mixer for 1 h at room temperature. Beads were then centrifuged at $3000 \times g$ for 2 min at 4°C and washed with the washing buffer (pH 8,

Table 1. Demographics of patients with and without a DS pregnancy at the time of screening

	DS	CTR
Maternal age (years)	31.9 ± 5.3	33.1 ± 5.1
Gestational age (weeks)	15.4 ± 1.1	15.5 ± 1.1
Abnormal maternal screen	70%	15%

50 mM Tris HCl, 150 mM NaCl, 0.1% Tween 20) three times. Immunoprecipitated complex was recovered by resuspending the pellet with 25–50 μL of 2 × loading buffer. Samples were boiled at 95–100°C for 5 min to denature the protein and separate it from the protein-A/G beads, then centrifuged and the supernatant used for further analysis.

2.12 Post Western blot immunochemical detection of protein carbonyl levels

The carbonyl levels of ApoA1 were measured by Western blot to validate the redox proteomics results. Following the electroblotting procedure, the nitrocellulose membranes were equilibrated in 20% methanol for 5 min. Membranes were then incubated in 2 N HCl for 5 min. The membranes were next incubated in 0.5 mM DNPH solution exactly 5 min. The membranes were washed three times in 2 N HCl and five times in 50% methanol (5 min each wash). After post-Western blot derivatization, the immunochemical detection and measurement of carbonyl levels of ApoA1 were similar to that for total protein carbonyl level detection described above.

2.13 Statistical analysis

Two-sided Student's *t*-tests were used to analyze differences in protein levels between DS and CTR. A *p*-value of less than 0.05 was considered statistically significant. The significance of the change in carbonylation of specific proteins in the proteomics study was evaluated via nonparametric Mann–Whitney–Wilcoxon test. $p < 0.05$ was considered statistically significant.

3 Results

Ten AF samples were obtained from confirmed DS pregnancies and ten from normal euploid singleton pregnancies. Care was taken to match both maternal and gestational age, to rule out any confounding influence of these two parameters (Table 1).

3.1 Protein oxidation and lipid peroxidation

To assess the extent of protein oxidation in DS AF, a set of circulating markers of OS were evaluated by slot-blot analysis. Protein carbonyls were significantly increased in AF from women carrying DS fetuses with respect to AF from women carrying normal fetuses ($p < 0.05$: Fig. 1). As expected (Fig. 1) also HNE-protein adducts were significantly increased ($p < 0.05$) in the pathological samples (more than 1.5-fold respect to controls), indicating that the lipid peroxidation pathways were enhanced even at the fetal stage in DS.

3.2 Glutathione and Trx determination

In order to test the efficiency of GSH and Trx thiol-disulfide reductive systems, total and oxidized GSH and Trx contents were assayed in DS and normal AF. Glutathione assay results, shown in Table 2, indicated a decrease of total glutathione and an increase of GSSG levels in DS AF with respect to controls ($p < 0.05$).

Consistent with this result, Trx levels were found to be much lower (50%) in DS AF with respect to control ($p < 0.05$; Fig. 2), confirming a loss of thiol-reducing system.

3.3 Heat shock response

By Western blot experiments, three different heat shock proteins were evaluated: Heat shock protein 70 (HSP 70), glucose regulated protein78 (Grp 78) and heme oxygenase 1 (HO-1, also called HSP 32). As shown in Fig. 3, the heat shock response was enhanced in AF from DS pregnancies

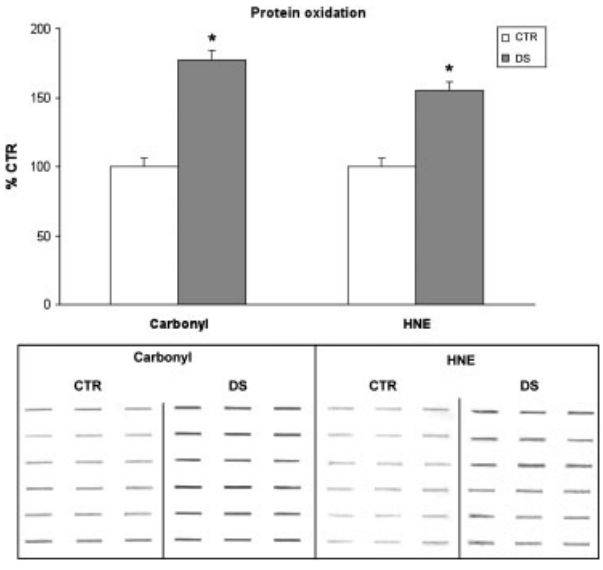


Figure 1. OS as indexed by protein carbonylation (left) and protein-bound HNE (right). Slot-blot showed a significant increase in the levels of protein carbonyls in AF from women carrying DS fetuses ($n = 10$) compared to AF from women carrying normal fetuses ($n = 10$) was found. These data are presented as means ± SEM. Statistical analyses employed Student's two-tailed *t*-test. $^*p < 0.05$ versus CTR.

Table 2. Total glutathione (GSHt) and oxidized glutathione (GSSG) levels in AF from normal (CTR) or DS fetuses

	GSHt (μM)	GSSG (μM)	GSSG/GSHt ratio
CTR	1.69±0.10	0.152±0.030	0.089±0.015
DS	1.38±0.16	0.173±0.027	0.125±0.017

Reported values represent the mean±SEM of three independent experiments. Statistical analyses employed Student's two-tailed *t*-test. $p<0.05$ DS versus CTR.

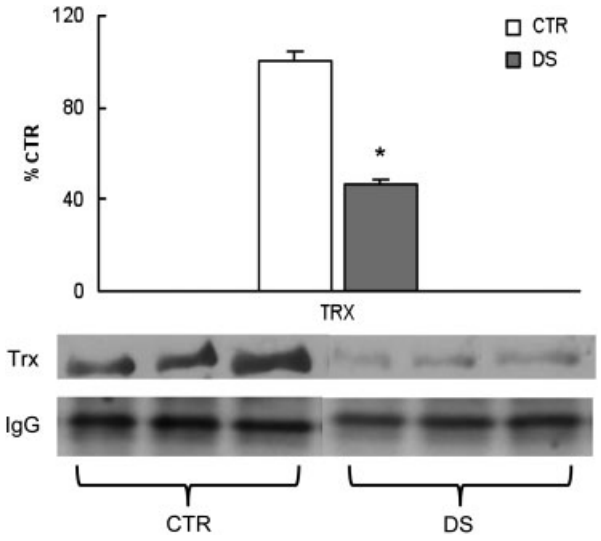


Figure 2. Western blot analysis showed decreased expression level of Trx in DS AF with respect to controls. Immunoblots were scanned by densitometry and all values were normalized to IgG levels. Densitometric values are given as percentage of CTR values ($n=3$) and represent the mean±SEM of three independent experiments. $^*p<0.05$ versus CTR.

with respect to normal pregnancies, with increments of 2.1-, 2.8- and 2.5-fold, for HSP 70, Grp 78 and HO-1, respectively.

3.4 Redox proteomics for the identification of carbonylated proteins

In comparison with CTR, DS fetuses have shown ten proteins significantly more oxidized. Figure 4 shows representative 2-D gels of AF from normal fetus (left top) and AF from DS fetus (left bottom) And a representative 2-D blots of AF from normal fetus (right top) and AF from DS fetus (right bottom). These proteins are ceruloplasmin (CP), serotransferrin, complement component C9, α-1B-glycoprotein, kininogen-1, zinc-α-2-glycoprotein (ZAG), collagen α-2(I) and α-1(V) chain, insulin-like growth factor-binding protein 1 (IGFBP-1), ApoA1 and retinol-binding protein 4 (RBP4).

Table 3 shows the proteins successfully identified by mass spectrometry along with the peptide hits, sequence coverage,

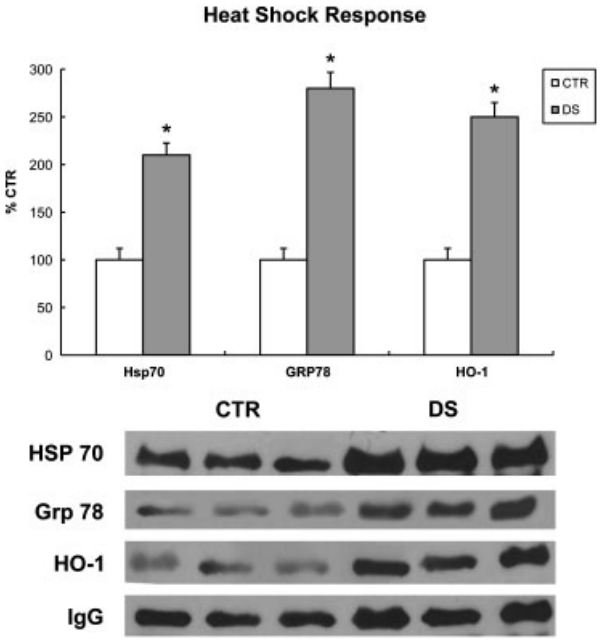


Figure 3. HSP 70, Grp 78 and HO-1 levels were measured by Western blot analysis using specific antibodies. Immunoblots were scanned by densitometry and all values were normalized to IgG levels. Densitometric values are given as percentage of CTR values ($n=3$) and represent the mean±SEM of three independent experiments. $^*p<0.05$ versus CTR.

Mw and p*I* values and the increase in specific carbonyl levels, indexed as fold oxidation compared with controls.

Although the Mowse score was not significant for collagen α-2(I) chain and collagen α-1(V), the putative identications were confirmed by MS/MS (Supporting Information).

Specific oxidation of ApoA1 was evaluated by post Western immunodetection of carbonyl levels of immunoprecipitated protein, confirming the proteomics results (Fig. 5).

4 Discussion

DS is characterized by altered genetic heritage, i.e. trisomy21, and the effect of this abnormal chromosomal condition leads to a wide variability of phenotypes. Although OS is known to play a major role in DS [6], the molecular mechanisms underlying the pathological alterations and how the oxidative imbalance can contribute to the clinical manifestation of DS are not yet known [1]. Thus, the present study aims to evaluate a set of OS biomarkers in DS AF that could prove in vivo the occurrence of accelerated ageing phenomena including the development Alzheimer's-like dementia and other redox-related pathologies seen in DS.

The levels of both protein carbonyls and protein-bound HNE were found significantly increased in AF of DS fetuses in comparison with control. These results are consistent with those reported by Odetti et al. [25] showing enhanced protein carbonylation (besides lipoperoxidation and glycoxidation

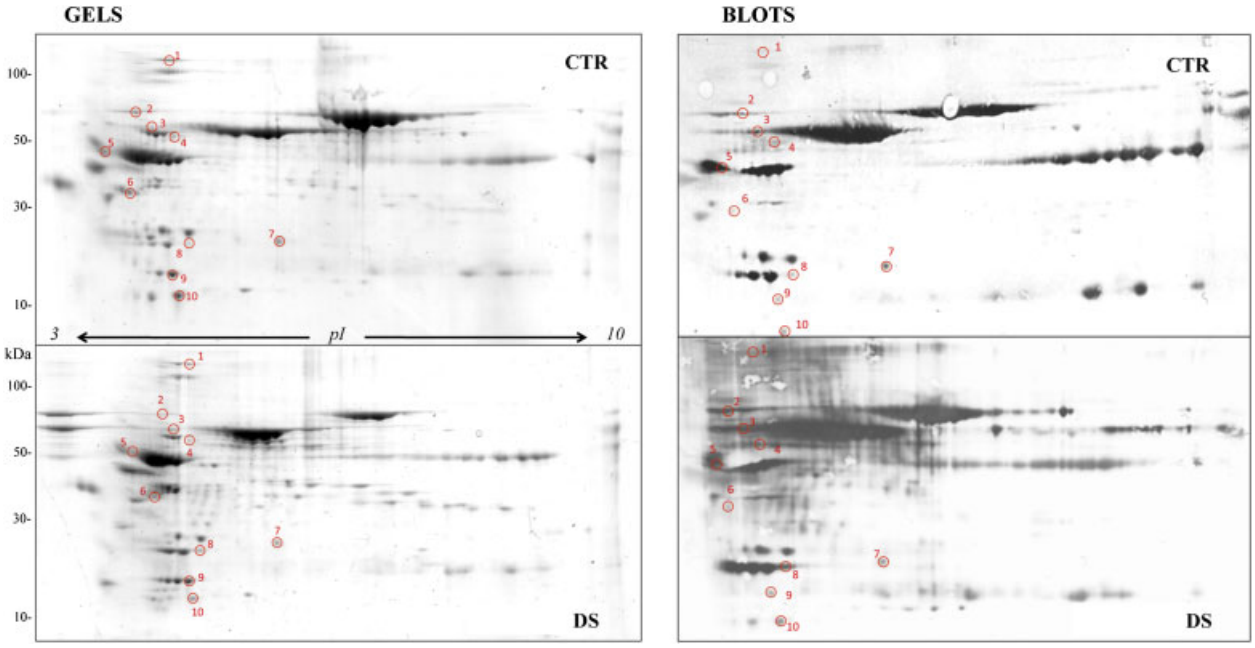


Figure 4. Left: 2-D maps of AF samples from CTR (top) and DS (bottom) fetuses. Protein (200 μg) extracts were analysed in first dimension (pH 3–10 linear IPG); second dimension was performed on slab gel (12% gradient SDS-PAGE). Protein detection was achieved using Biosafe Coomassie staining; Right: 2-D carbonyl immunoblots from control (top) and DS AF (bottom). The spots showing significant increased carbonyl levels between cases and control are labeled. Relative change in carbonyl immunoreactivity, after normalization of the immunostaining intensities to the protein content, was significant for ten spots. See text.

increases) in the cerebral cortex of Down fetuses. In addition, DS neurons exhibit a consistent increase in intracellular ROS and elevated levels of lipid peroxidation that precede neuronal death [26].

OS occurs also as a consequence of the body's inability to detoxify reactive intermediates. Several reports highlight the key role of sulfydryl groups in response to OS [27, 28]. GSH significantly contributes to the detoxification of hydroxyl radical. Besides being an antioxidant, reduced GSH is a molecule of highest importance in modifying proteins in response to the intracellular redox state. Therefore, the intracellular pool of GSH is thought to reflect the oxidative pressure within a cell [29, 30]. Trx is a general protein disulfide reductant; the Trx/Trx reductase system takes an active part in limiting OS damage, either directly scavenging ROS and indirectly by protein–protein interactions, thus playing an essential role in maintaining intracellular redox status. GSH and Trx systems also regulate signal transduction via intracellular redox balance [31]. Moreover Trx act in concert with GSH reductase to maintain GSH in the reduced form.

The current investigation is the first report showing an alteration of both Trx and GSH systems, i.e. decrease of reduced Trx and total glutathione and a mild increase of GSSG in DS pregnancies with respect to normal pregnancies. A reduction of these antioxidant systems leads to increased OS, which have been shown to be an active part of neurodegenerative phenomena. Accordingly, Gulesserian et al. [32] suggested that OS in fetal DS results from low levels of reducing agents, rather than of SOD-1 overexpression [33]. Indeed, it is important to note that, in contrast to adult brain, fetal DS neurons were not found to overexpress SOD-1, and therefore the impaired oxidative status may occur primarily as a consequence of the low levels of reducing agents and enzymes involved in the removal of ROS [32].

OS induces defensive reactions, involving induction of scavenging and repair systems. In the present work, we reported results demonstrating the activation of heat shock response in DS AFs. We found that some HSPs, namely HSP 70, Grp 78 and HO-1, were upregulated in AF from Down pregnancies compared to normal ones. HSP 70 is a functional chaperone triggering a cascade of intracellular cytoprotective events. Grp 78, is a member of the HSP 70 family of proteins which function as molecular chaperones. During the ER stress response, Grp 78 binds misfolded proteins and translocates then through ER membranes for their proteasomal degradation [34]. The involvement of HO-1, in OS-related disorders, including AD, has gained considerable importance in recent years [35]. Induction of HO-1 catabolizes heme and generates carbon monoxide and biliverdin and subsequently bilirubin, a potent antioxidant and anti-inflammatory agent. The expression of HO-1 is upregulated during OS, as well as by GSH depletion [36–38]. Our results on AF are in line with Lubec and co-workers who reported differential expression of HSPs in the brains of patients with DS [39, 40], and in cerebral cortex of DS fetuses [41]. The activation of the HSP response likely represents a cytoprotective mechanism towards an OS

Table 3. Summary of the proteins identified by redox proteomics that are increasingly carbonylated in the DS samples versus control

Spot	Protein name	Swiss-Prot code	Mowse score	Mw/*pI* theoretical	Matched peptides	Sequence coverage %	*p* value	Identification	Fold oxidation
1	CP	P00450	183	122 983/5.44	15/16	15	<0.05	MS	8
2	Serotransferrin	P02787	113	79 280/6.81	8/9	13	<0.05	MS	16.6
3	Complement component C9	P02748	70	64 615/5.43	5/12	10	<0.05	MS	1.48
4	α-1B-glycoprotein	P04217	168	54 809/5.58	10/24	39	<0.05	MS	5.9
5	Kininogen-1	P01042	100	72 996/6.34	7/8	11	<0.05	MS	28
6	ZAG	P25311	122	34 465/5.71	10/22	39	<0.05	MS	5.6
7	Collagen α-2(I) chain	P08123	49	129 723/9.08	5/9	4	<0.05	MS, MS-MS	7.3
	Collagen α-1(V) chain	P20908	34	184 131/4.94	3/9	3			
8	IGFBP-1	P08833	77	28 912/5.11	4/7	19	<0.05	MS	4.7
9	ApoA1	P02647	189	30 759/5.56	17/27	46	<0.05	MS	2.84
10	RBP4	P02753	102	23 337/5.76	6/10	44	<0.05	MS, MS-MS	5.17

For each protein, the carbonyl immunoreactivity/protein expression values were averaged ($n = 8$) and expressed as fold oxidation compared to control. The *p*-value listed is the significance of increased carbonyl levels relative to control samples with $p<0.05$. Probability-based Mowse scores were estimated by comparison of search results against estimated random match population and were reported as $-10 \times \text{Log10}(p)$, where *p* is the absolute probability. A Mowse score above 65 indicates a significant identity when searching in the total database.

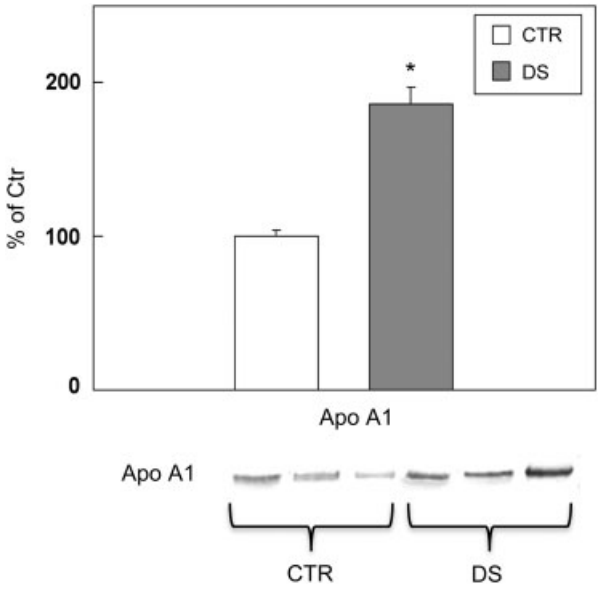


Figure 5. Increased carbonyl level of ApoA1. Data represent the alteration of the ApoA1 carbonyl levels in DS samples compared to control using traditional immunochemical detection. Error bars indicate SEM for three samples in each group. Measured values are normalized with the mean of the control samples. $^{*}p<0.05$.

condition occurring concomitantly with the trisomy of chromosome 21.

In order to identify oxidatively modified proteins in the AF of the DS group, which might be responsible of the increased susceptibility of DS patients to develop multiple pathologies, we used a redox proteomics approach. Previous studies from Tsangaris et al. [42] demonstrated the feasibility of proteomic approaches in identifying proteins differentialy expressed in AF from DS fetuses compared with controls. Accordingly, in our expression proteomic study (data not shown), we found that AMBP and procollagen III were upregulated in DS versus CTR [42], moreover differential expression of transtyrethin, serotansferrin, ApoA1, gelsolin and α-1B-glycoprotein were in line with results shown by Park et al. [43].

In addition, other groups employed proteomic technology in prenatal diagnosis to identify putative biomarkers for early and accurate detection of fetuses at risk of chromosomal abnormalities [44–46].

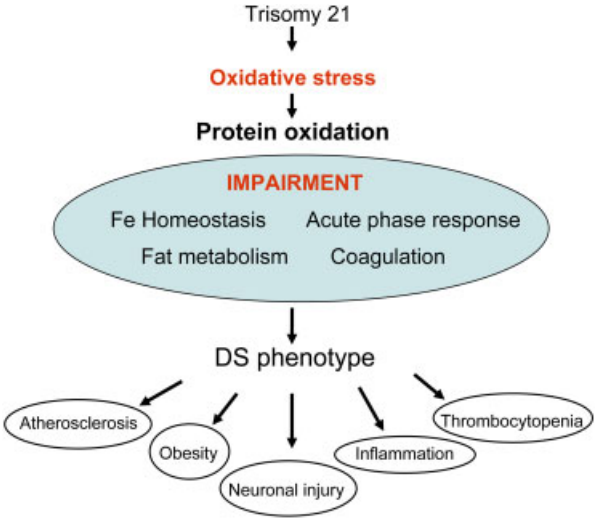
Our results showed that ten proteins had increased carbonylation in AF from women carrying DS fetuses: these proteins are involved in iron homeostasis, lipid metabolism and inflammatory response. Deregulation of these pathways might contribute to or exacerbate degenerative phenomena manifested in DS (Scheme 1).

4.1 Fe homeostasis: CP and transferin

CP is a multicopper enzyme containing around 95% of circulating copper. Its main function is to form water, reducing molecular oxygen, and to oxidize ferrous ions to the less toxic ferric form, without releasing ROS. This enzymatic activity confers CP a relevant antioxidant power and a significant role in iron homeostasis because ferrous ions, entering Fenton reactions, take part in an OS cascade [47]. Although fundamental for normal brain activity, Fe^{2+} may induce neuronal injury by catalyzing the conversion of hydrogen peroxide into highly reactive radicals. During exposure to OS, it has been suggested that substantial CP inactivation may occur and free copper ions could be released [48, 49]. Therefore, damaged CP may cause in turn the propagation of free radical-mediated damage to other macromolecules upon exposure to OS. Iron homeostasis is mainly regulated by transferrin (Tf), a glycoprotein organized in two domains containing a Fe-binding site.

Clinical Relevance

Down syndrome is the most common genetic cause (trisomy 21) of mental retardation which manifests with unpredictable feautures leading to several differences among individual's phenotype. Although OS is considered to be involved DS pathogenesis, a direct cause-and-effect relationship between the accumulation of oxidatively mediated damage and clinical manifestation of DS are not yet strongly established. Several studies have been performed on Down fetal brains, while few data are available on AF, which is a more reliable index of the physiological condition of the fetus. Thus, this study aims to evaluate a set of OS biomarkers in DS AF that could correlate with redox-related complications seen in DS such as accelerated ageing and the development Alzheimer's-like dementia. This observational study indicates that OS occurs early in the DS pathogenesis and could play a relevant role in the output of deleterious DS phenotypes characterized by abnormal development and, a few decades later, AD-like neuropathology.



Scheme 1. Putatitve network of altered pathways implicated in DS phenotype as a consequence of increased OS.

In serving this function, Tf accepts ferric ions, oxidized by CP, and transports them to other cells. Iron-mediated damage has been implicated in several conditions affecting the central nervous system. Increased carbonyl levels of both CP and transferrin in DS AF might be responsible of impaired Fe storage, which possibly initiates multiple redox reactions that damage living cells via various pathways. Thus, resulting in an exacerbated OS condition that contributes to the development of deleterious DS phenotypes.

IGFBP-1 is a secreted protein that binds to IGF in extracellular environments. IGFBP-1 regulates minute-to-minute bioavailability of IGFs in the circulation and inhibits IGF actions [50]. IGFs are a family of mitogenic polypeptides that have been shown to be important determinants of fetal growth during human pregnancy[51–53]. Interestingly, selective deficiency of IGF-I in the serum has been seen in patients with DS older than 2 years [54]. We hypothesize that IGFBP-1 oxidative modification in the early phase of the disease (i.e. fetal stage) contributes to abnormal development of the brain with relevant implication to cognitive disabilities characteristic of DS. Indeed, in DS brain neurodevelopmental impairment seems to be a key determinant of cognitive defects. The present findings extend and reinforce the observation that levels of circulating insulin, IGF-I and IGFBPs are altered in many types of human neurodegenerative diseases, including major illnesses such as Alzheimer disease or stroke as well as inherited neurodegenerative diseases [55, 56].

4.2 Fat metabolism: ZAG, RBP4 and ApoA1

ZAG and RBP4 have been recently classified as adipokines involved in the etiopathology of obesity-related disorders. ZAG may directly stimulate lipolysis through interaction with a β3-adrenoreceptor, suggesting a role in lipid catabolism, coupled with an increase in energy expenditure [57]. RBP4 is a transport protein for retinoids such as vitamin A in the blood. [58] It is mainly produced in the liver but has recently been identified as an adipokine [59], which is able to modulate glucose metabolism. This factor, possibly working in concert with retinol, impairs insulin signaling in muscle, inhibiting glucose uptake and interferes with insulin-mediated suppression of glucose production in the liver, causing blood glucose levels to rise. We found increased carbonyl levels of both ZAG and RBP4. This result might suggest that altered function of these adipokines is implicated in the compliance of fat metabolism observed in DS patients. In fact, young DS patients are often obese with lipid metabolic problems [60].

Consistent with these results, we also found increased carbonyl levels of ApoA1. It has been demonstrated that apolipoproteins play an important role in lipoprotein metabolism in the CNS. ApoA1 is the major apolipoprotein associated with HDL, and apoA1 deficiency in humans leads to a phenotype of low plasma HDL levels and premature atherosclerosis [61]. ApoA1 knockout mice also exhibit a marked reduction in plasma HDL levels [62, 63] that is reflected in levels of total plasma cholesterol. The finding of increased oxidation of ApoA1 is in line with recent reports highlighting that ApoA dysfunction may be linked to increased susceptibility to cognitive impairment [64].

4.3 Acute-phase response: Complement C9, α-1B-glycoprotein, collagen α-1(V) chain

These proteins are known to be part of the acute-phase response and they may have a protective function for the developing fetus. As these proteins are present in AF, it is possible that they may prevent intra-amniotic infection and the subsequent complications that can result. However, once they are oxidized, they might loose their biological function and therefore not be able to achieve their role in protecting the fetus. These results are in line with those reporting increased susceptibility of Down subjects to acquired immunodeficiency with also increased incidence of autoimmune diseases [65, 66].

4.4 Blood coagulation: Kininogen (HK)

HK was also oxidized in DS AF. HK, together with prekallikrein and factor XII, comprise the contact system of coagulation [67]. In addition, HK also plays important roles distinct from the initiation of coagulation via interactions with platelets, neutrophils, monocytes, macrophages and endothelial cells. Through these interactions, HK modulates cellular adhesion, leukocyte extravasation and vascular permeability [68, 69]. This result is consistent with previous studies showing that the properties of kininogens were altered by oxidation of their methionine residues by ROS [70]. One abnormal finding in newborns with DS is a low platelet count. Rarely, the platelet count may be so low that transfusions may be needed to prevent bleeding problems. The reason for the thrombocytopenia is unknown. Considering that HK can serve as an important regulator of the early stages of platelet stimulation by thrombin, it is reasonable to speculate that oxidation of HK may lead to a temporary lack of regulation of the platelet precursor cells in neonates with DS and might explain why DS patients have an increased risk of leukemia.

In conclusion, our study demonstrates that OS occurs early in the pathogenesis of DS and might contribute to the severity of DS phenotypes. Accordingly, several markers of OS have been found to be significantly increased in the AF. We suggest that the increased oxidation of specific proteins might lead to the impairment of multiple cellular pathways, which are involved in DS clinical outcomes. Indeed, the proposed scenario could correlate with some characteristic features of DS including early ageing [71], neurological and cognitive impairment [72], increased risk for cancer and immunodeficiency [73, 74].

This study contributes to provide the groundwork for developing new pharmacological and/or nutritional interventions, based on antioxidant compounds, in DS patients since the earliest life stages for the prevention, or at least, slowing of the multiple complications observed in DS.

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