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Low-molecular weight compounds in human seminal plasma as potential biomarkers of male infertility

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STUDY QUESTION: Is the determination of antioxidants, oxidative/nitrosative stress-related compounds, purines, pyrimidines and energy-related metabolites in human seminal plasma of utility to evidence biomarkers related to male infertility?

SUMMARY ANSWER: The determination of 26 metabolites in seminal plasma allowed to evidence that 21/26 of them are biomarkers of male infertility, as well as to calculate a cumulative index, named Biomarker Score, that fully discriminates fertile controls from infertile patients and partially differentiates infertile without from infertile with spermiogram anomalies.

WHAT IS KNOWN ALREADY: Epidemiological studies indicated that a male factor is involved in ~50% of cases of pregnancy failure, with a significant percentage of infertile males having no alterations in the spermiogram. Further laboratory analyses of male infertility are mainly dedicated only to gross evaluations of oxidative stress or total antioxidant capacity.

STUDY DESIGN, SIZE, DURATION: Seminal plasma of 48 fertile controls and 96 infertile patients (master group), were collected from September 2016 to February 2018. A second group of 44 infertile patients (validation group) was recruited in a second, independent centre from September 2017 to March 2018. Samples were analysed in blind using a 'Redox Energy Test' to determine various low-molecular weight compounds, with the aim of finding metabolic profiles and biomarkers related to male infertility.

PARTICIPANTS/MATERIALS, SETTING, METHODS: In all seminal plasma, 26 water- and fat-soluble compounds (related to anti-oxidant defences, oxidative/nitrosative stress, purine, pyrimidine and energy metabolism) were analysed using high-performance liquid chro-matographic methods. According to spermiogram, infertile patients of both groups were also categorized into normozoospermic (N, no anomalies in the spermiogram), or into the subgroup including all patients with anomalies in the spermiogram (asthenoteratooligozoospermic ATO + asthenozoospermic A + teratozoospermic T + oligozoospermic O).

MAIN RESULTS AND THE ROLE OF CHANCE: In the master group, results indicated that 21/26 compounds assayed in seminal plas-ma of infertile males were significantly different from corresponding values determined in fertile controls. These 21 compounds constituted the male infertility biomarkers. Similar results were recorded in patients of the validation group. Using an index cumulating the biochemical seminal plasma anomalies (Biomarker Score), we found that fertile controls had mean Biomarker Score values of 2.01 ± 1.42, whilst infertile patients of the master and of the validation group had mean values of 12.27 ± 3.15 and of 11.41 ± 4.09, respectively ($P < 0.001$ compared to controls). The lack of statistical differences between the master and the validation groups, in both the metabolic profiles and the Biomarker

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Score values, allowed to pool patients into a single cohort of infertile males. The Biomarker Score values showed that fertile controls and infertile males clustered into two distinct groups. Infertile patients without (N, $n = 42$) or with (ATO + A + T + O, $n = 98$) spermiogram anomalies differed in some biomarkers (ascorbic acid, all-trans retinol, α-tocopherol, cytidine, uridine, guanine). These differences were reinforced by distribution frequencies and posterior probability curves of the Biomarker Score in the three groups.

LIMITATIONS, REASONS FOR CAUTION: Results were obtained in relatively limited number of human seminal plasma samples. Using the 'Redox Energy Test' it was possible to associate specific metabolic profiles and values of the Biomarker Score to fertile controls or infertile males. However, it was not possible to evaluate whether the different anomalies of the spermiogram are associated with specific metabolic profiles and values of the Biomarker Score.

WIDER IMPLICATIONS OF THE FINDINGS: The 'Redox Energy Test', coupled with the Biomarker Score that cumulates the biochemical characteristics of seminal plasma into a single index, evidenced a set of low-molecular weight biomarkers potentially useful in the laboratory management of male infertility.

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Key words: male infertility / human seminal plasma / oxidative/nitrosative stress / mitochondrial dysfunction / biomarkers

Introduction

Infertility, defined as the inability to achieve pregnancy after at least 12 months of regular intercourse, is a multifactorial phenomenon, with both males and females implicated in the cause, approximately affecting 48.5 million couples worldwide (15% of reproductive-aged couples) (Pfeifer *et al.*, 2013). Epidemiological studies have indicated that a male factor is involved in ~50% of cases of pregnancy failure, with an exclusive responsibility in the 30% of all cases and a co-contributing female factor in the remaining 20% (Ko *et al.*, 2014; Winters and Walsh, 2014).

Since pathophysiological, environmental, genetic and life-style factors are involved, male infertility is considered a multifactorial disease (Cui *et al.*, 2016) and it is generally divided into four groups: extratesticular, testicular, pre-testicular and idiopathic (Bhasin, 2007; Pfeifer *et al.*, 2013; Ko *et al.*, 2014; Winters and Walsh, 2014; Cui *et al.*, 2016). This last group, in whom the primary cause is not clearly manifest, accounts for the 30–40% of cases of male infertility (Ray *et al.*, 2012).

An increasing number of studies has been dedicated to evaluate the role of oxidative stress in the development of male infertility. Oxidative stress, defined as an imbalance between reactive oxygen species (ROS) production and cell antioxidant defences, has been associated with the pathobiological processes of male infertility (Ko *et al.*, 2014; Agarwal *et al.*, 2014a). Notwithstanding, physiological ROS levels play important roles in the proper exploitation of various sperm processes (Aitken *et al.*, 2004; Suarez, 2008; Agarwal *et al.*, 2014b). The excess production of these harmful compounds induces a condition of oxidative stress triggering irreversible modification to biologically fundamental molecules of spermatozoa (Aitken *et al.*; 2016; Bisht and Dada, 2017; O'Flaherty and Matsushita-Fournier, 2017). Increased ROS production is frequently caused by dysfunctional mitochondria, characterized by decreased phosphorylating capacity with consequent ATP decrease and cell energy deficit, thereby linking redox and energy metabolism (Cassina *et al.*, 2015).

In various biological context, it has been demonstrated that oxidative stress is also associated with nitrosative stress (Caruso *et al.*, 2017; Kanaan and Harper, 2017), caused by an excess production of reactive nitrogen species (RNS). ROS and RNS are scavenged by low-molecular weight antioxidants such as ascorbic acid, uric acid and reduced glutathione (Regoli and Winston, 1999). To date, the concentrations of the full pattern of low-molecular weight antioxidants in seminal plasma of fertile and infertile males is still unknown. Furthermore, studies to connect the seminal plasma concentrations of compounds representative of energy metabolism and mitochondrial function (such as purines and pyrimidines) with male infertility are lacking.

With the aim to find new biochemical biomarkers and specific metabolic profiles related to male infertility, in this study we carried out the analysis of hydrophilic and hydrophobic antioxidants, of metabolites related to energy metabolism and of compounds related to oxidative/nitrosative stress in human seminal plasma of fertile control subjects and in two independent cohorts of infertile patients.

Materials and Methods

Ethical approval

The study was approved by the local Institutional Ethical Committees of the centres involved (Alma Res Fertility Centre, University of Catania, Azienda Ospedaliera S. Camillo-Forlanini) and was conducted according to the Declaration of Helsinki for Medical Research involving Human Subjects. Informed written consents were obtained from each control and patient enroled in this study.

Patient population

One group of infertile patients (master group, $n = 96$), was recruited at the Alma Res Fertility Centre (Rome, Italy) from September 2016 to February 2018. A second group of patients (validation group, $n = 44$), recruited at the Azienda Ospedaliera S. Camillo-Forlanini (Rome, Italy) from September 2017 to March 2018, was used as a second, independent sample of infertile males.

Patients enroled in the two groups were unable to obtain pregnancy after 2 years of unprotected sexual intercourses. They were clinically assessed, at the time of semen donation, to evaluate the presence of

infections, varicocele, cryptorchidism, obstructions, testicular tumours and any systemic disease. These pathological states, as well as chromosomic aberrations, were considered as exclusion criteria. Female partner of each patient of both groups underwent gynaecological assessment, including hormone analysis, hysterosalpingography and transvaginal ultrasound to evaluate uterine cavity and antral follicle counts. This permitted the exclusion of patients of which the female factor was involved in the couple's infertility.

A group of fertile healthy volunteers was used as the control group (n = 48). Normal semen parameters and proven fertility in the previous 6 years were used as the only inclusion criteria.

To reduce confounding factors, all participants (including controls) were interviewed to assess they had a similar dietary pattern and lifestyle (all non-smokers, no one with alcohol dependence, mild-to-moderate physical activity). Additionally, none of them was taking oral adjuvant/nutraceutical supplements during the 3 months before semen donation.

Collection of seminal liquid, analysis of spermiogram and preparation of seminal plasma

Semen specimens were produced by masturbation after a recommended period of 2–5 days of sexual abstinence. After a complete liquefaction on the bench at 37°C for 20 min, spermiogram to determine sperm motility, concentration and morphology, according to WHO guidelines (World Health Organization, 2010), was carried out.

The complete freshly and liquefied semen samples were centrifuged for 15 min at 1860 *g*, to remove spermatozoa and other cells from seminal plasma. The seminal plasma was immediately withdrawn and processed for the HPLC analysis, named 'Redox Energy Test', of selected low-molecular weight metabolites. The biochemical analyses of samples were blinded.

Processing of samples and reversed phase HPLC assay of fat-soluble vitamin and antioxidants, and hydrophilic low-molecular weight metabolites

Each seminal plasma sample was divided into two aliquots of 300 µl each. One aliquot was processed according to a procedure recently described in detail elsewhere (Lazzarino *et al.*, 2017a). Briefly, one volume of seminal plasma (300 µl) was added to two volumes (600 µl) of HPLC-grade CH_3CN (Carlo Erba, Milan, Italy). After vigorous vortexing, samples were incubated at 37°C for 1 h in a water bath and then centrifuged at 20 690 *g* for 15 min at 4°C to precipitate proteins. Clear supernatants were directly injected (200 µl) onto the HPLC column for the analysis of fat-soluble vitamins and antioxidants (Lazzarino *et al.*, 2017a).

The second aliquot of seminal plasma (300 µl) was deproteinized with HPLC-grade CH_3CN (600 µl), immediately centrifuged (to pellet precipitated proteins) and supernatants washed with HPLC-grade chloroform (to remove organic solvent), as previously described (Tavazzi *et al.*, 2005). The aqueous phase (50 µl) was diluted 10 times with HPLC-grade water and then directly injected (100 µl) onto the HPLC column for the analysis of the compounds of interest (Tavazzi *et al.*, 2005; Chaleckis *et al.*, 2016).

HPLC analysis of metabolites of interest

In the organic solvent extracts, *all-trans*-retinoic acid, *all-trans*-retinol, γ-tocopherol, α-tocopherol, astaxanthin, lutein, zeaxanthin, *trans*-β-apo-8′-carotenal, β-cryptoxanthin, lycopene, α-carotene, β-carotene and coenzyme Q_{10} were separated and quantified using a Hypersil Gold RP C18, 100 × 4.6 mm, 5 µm particle size column, provided with its own guard column (Thermo Fisher Scientific, Rodano, Milan, Italy), as previously described (Lazzarino *et al.*, 2017a).

In the aqueous extract, water-soluble antioxidants (reduced glutathione (GSH) and ascorbic acid), biomarkers of oxidative/nitrosative stress (malondialdehyde (MDA), 8-hydroxy-2′-deoxyguanosine (8-OHdG), nitrites and nitrates), purines (hypoxanthine, xanthine, uric acid, inosine, adenosine, guanine and guanosine), pyrimidine (uracil, β-pseudouridine, uridine, cytidine, cytosine and orotic acid) and creatinine were separated and quantified as described in detail elsewhere (Tavazzi *et al.*, 2005). This sensitive and reliable chromatographic method was used to measure similar pattern of metabolites in various biological samples (Lee *et al.*, 2013; Bracko *et al.*, 2014; Amorini *et al.*, 2016; Bordbar *et al.*, 2016; Eidt *et al.*, 2016; Macchiaiolo *et al.*, 2017; Wang *et al.* 2017; Lazzarino *et al.*, 2017b; Fresta *et al.*, 2018). Aqueous extracts were diluted with HPLC-grade water (1:10, v/v) and loaded (100 µl) onto a Hypersil C-18, 250 × 4.6 mm, 5-µm particle size column, provided with its own guard column.

Selectivity, specificity, linearity, limits of detection/quantification and recovery efficiencies of both extraction and detection methods were fully described in the previous analytical studies (Tavazzi *et al.*, 2005; Lazzarino *et al.*, 2017a). For both analyses, the HPLC apparatus consisted of a Spectra System P4000 pump, equipped with a highly sensitive 5 cm light-path flow cell UV6000LP diode array detector, setup for acquisition between 200 and 550 nm wavelengths. Data acquisition and analysis were performed using the ChromQuest software package provided by the HPLC manufacturer (Thermo Fisher Scientific, Rodano, Milan, Italy).

Identification and quantification of the compounds of interest in chromatographic runs of seminal plasma samples were obtained by comparing retention times and absorption spectra of different peaks with those of runs of standard mixtures containing true compounds with known concentrations. In the final calculations in seminal plasma, concentrations of astaxanthin, lutein, zeaxanthin, *trans*-β-apo-8′-carotenal, β-cryptoxanthin, lycopene, α-carotene and β-carotene were summed and reported hereinafter as total carotenoids.

Statistical analysis

Statistical analysis was performed by using the Statistical Package for Social Science (SPSS), release 15.0. Data were first analysed for normality of distribution using the Kolmogorov–Smirnov test. Continuous variables were expressed as mean ± SD, categorical variables displayed as frequencies and the appropriate parametric (Student's *t*-test or ANOVA) or non-parametric tests (Mann–Whitney *U* test, Kruskal–Wallis ANOVA, χ^2 test) were used to assess differences among fertile controls, the master and the validation groups of infertile males, as well as between the two infertile subgroups without and with anomalies in the spermiogran. A P value of <0.05 was considered statistically significant.

For each of the 21 compounds with the meaning of biomarker for infertility, we determined the 5 and 95% percentile of its seminal plasma concentration in control subjects. Subsequently, each biomarker of the entire population (fertile control and infertile patients) was stratified into two categories: (i) Normal (0) = biomarker concentration ≤95 or ≥ 5% percentile of controls and (ii) Positive (1) = biomarker concentration falling outside the 5 or 95% percentile of controls. Each subject was then associated to a specific biochemical pattern of markers positivity and to a 'Biomarker Score' (sum of the number of Positive categories), thus ranging from 0 (all biomarkers classified as 'Normal') to 21 (all biomarkers classified as 'Positive'). The schematic stepwise process to obtain the Biomarker Score is reported in Fig. 1.

Results

Table I summarizes the clinical features of fertile controls and of the two groups (master and validation groups) of infertile patients.

According to the results of the spermiogram, infertile patients were assigned to the subgroups without (N) or with spermiogram anomalies (ATO + A + T + O).

Evaluation of the biochemical quality of seminal plasma from fertile donors

Values of the compounds under evaluation in seminal plasma of control fertile subjects were used to determine their 5–95% percentiles (Table II). In controls, ascorbic acid was the most abundant antioxidant in seminal plasma whilst minimal concentrations of nitrites, nitrates and MDA, or undetectable levels of 8-OHdG, were recorded. A peculiar pattern of purines, pyrimidines and energy-related metabolites, with high uridine, guanine and guanosine values (2764.57 ± 766.04, 63.79 ± 15.31 and 69.46 ± 22.52 μmol/l, respectively) was determined in seminal plasma of controls.

The biochemical quality of seminal plasma in infertile males

The analysis of seminal plasma of the infertile males of the master group evidenced a high number of anomalies. Impressively, 21/26 compounds measured in these patients (Table III), namely ascorbic acid, *all-trans* retinoic acid, *all-trans* retinol, α-tocopherol, γ-tocopherol, total carotenoids, MDA, 8-OHdG, nitrites, nitrates, creatinine, cytosine, cytidine, uracil, β-pseudouridine, hypoxanthine, xanthine, uridine, inosine, guanine and guanosine, were significantly different from values detected in fertile donors ($P < 0.001$). To assess that differences between fertile controls and infertile males were not due to differences in the extraction efficiencies of these two categories of seminal plasma samples, we carried out a specific set of experiments. Briefly, four randomly selected seminal plasma from controls and infertile males (master group) were initially assayed to determine the concentrations of the different analytes of interests. Subsequently, an aliquot

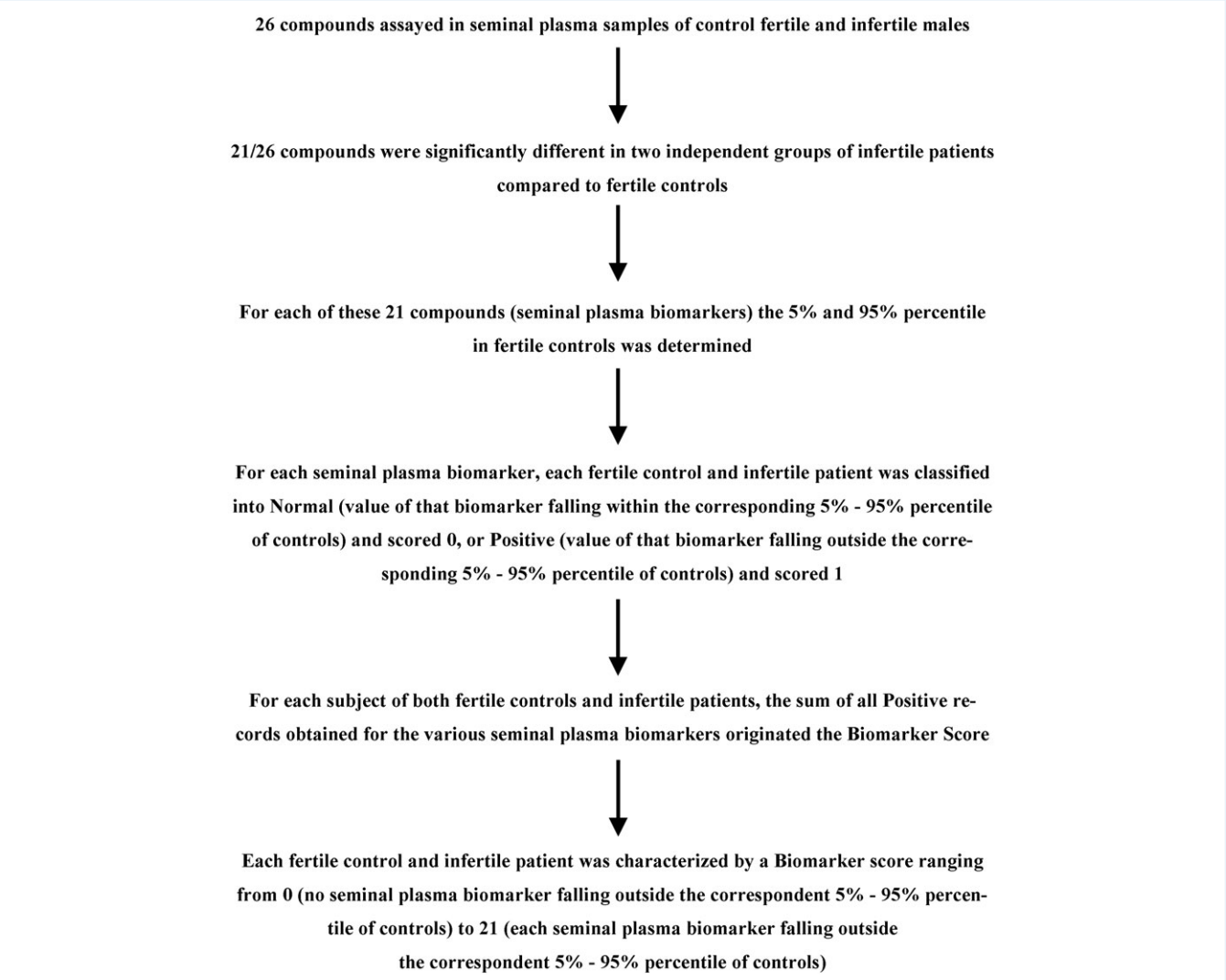

Figure 1 Schematic representation of the stepwise process occurring to obtain the Biomarker Score from the concentration values of the 21 seminal plasma biomarkers representative of antioxidants, oxidative/nitrosative stress-related compounds, purines, pyrimidines and energy-related metabolites, quantified by using a set of HPLC analyses named 'Redox Energy Test'.

Table I Morpho-functional parameters of semen on the spermiogram of fertile controls and infertile patients (master group).

	Age (years)	Sperm concentration (million/ml)	Total sperm motility (%)	Sperm morphology (% of normal spermatozoa)
Controls ($n = 48$)	38.63 ± 11.26	133.60 ± 40.12	86.91 ± 7.03	73.55 ± 9.84
Master group				
Infertile N ($n = 28$)	39.78 ± 7.48	51.22 ± 12.09[a]	60.43 ± 20.11[a]	14.38 ± 5.74[a]
Infertile ATO + A + T + O ($n = 68$)	40.84 ± 10.44	15.59 ± 9.26[a,b]	21.38 ± 12.71[a,b]	6.09 ± 2.64[a,b]
Validating group				
Infertile N ($n = 14$)	41.72 ± 15.11	62.78 ± 26.91[a]	67.49 ± 28.52[a]	18.48 ± 8.42[a]
Infertile ATO + A + T + O ($n = 30$)	42.06 ± 14.20	13.68 ± 7.45[a,b]	23.89 ± 10.16[a,b]	5.87 ± 1.99[a,b]

Values are the mean ± S.D. of fertile healthy controls and infertile patients, who were divided into two subgroups according to the absence (infertile N) or presence (infertile ATO + A + T + O) of anomalies in the spermiogram.
N = normozoospermic; ATO = asthenoteratooligozoospermic; A = asthenozoospermic; O = oligozoospermic; T = teratozoospermic.
No differences were observed in the values of the spermiograms of the master and the validating groups.
[a]Significantly different from controls, $P < 0.001$.
[b]Significantly different from the respective subgroup of N, $P < 0.001$.

of these samples was spiked with a standard mixture containing the compounds of interest with known concentrations. The spiked samples were then extracted and analysed to verify the recovery of each analyte. Results are summarized in Supplementary Information Tables S1–S3 and demonstrate that the efficiency of extraction is identical for control and infertile male seminal plasma samples. Therefore, the aforementioned compounds assumed the role of biomarkers of male infertility.

To corroborate the results obtained in infertile patients of the master group, we analysed seminal plasma in an independent validation group of 44 infertile males. Results clearly indicate that these patients have similar metabolic changes recorded in the master group, i.e. in both groups of infertile males the same 21 compounds significantly differed from corresponding values recorded in controls (Table IV). Furthermore, comparison of these two groups of infertile donors showed no differences in any of the compounds measured in seminal plasma. The lack of statistical differences between the master and the validation groups in any of the compounds measured in their seminal plasma allowed combining data of the two groups of infertile males into a single cohort of patients with infertility, for the further data analysis and statistical evaluations. Altogether, results of this validation group strongly reinforced the role of biomarkers of male infertility of these 21 compounds.

To verify whether the 21 candidate biomarkers of male infertility were differentially affected in patients without or with anomalies in the spermiogram, the pooled cohort of infertile males was divided into the two unrefined groups of infertile without (N) or with spermiogram anomalies (ATO + A + T + O). Idiopathic infertile N (Table V) had values of *all-trans* retinoic acid, *all-trans* retinol, α-tocopherol, γ-tocopherol, total carotenoids, MDA, 8-OHdG, nitrites, creatinine, cytosine, cytidine, uracil, β-pseudouridine, hypoxanthine, xanthine, guanine and guanosine significantly different from the corresponding values of fertile controls ($P < 0.01$). That is, the 'Redox Energy Test' of the seminal plasma found signatures of biochemical anomalies in those idiopathic patients who had no evident morpho-functional alterations in the spermiogram. In addition, infertile ATO + A + T + O had values of ascorbic acid, *all*-trans retinol, α-tocopherol, cytidine, uridine and guanine that were different both from controls and infertile N ($P < 0.01$).

The Biomarker Score as a cumulative index to assess the biochemical quality of seminal plasma in infertile males

Using the 5–95% percentiles of controls, values of each of the 21 metabolites (acting as biomarkers of male infertility) of each patient scored 0 (if falling within the range of normality) or 1 (if falling outside the range of normality). In both fertile and infertile males, a cumulative Biomarker Score was obtained as the sum of the different metabolite scores (Fig. 1). These values ranged from 0 (each metabolite scoring 0) to 21 (each metabolite scoring 1). In the preliminary analysis of the data, it was determined that the dispersion of the Biomarker Score values showed a normal distribution in both control and each group of infertile patients.

The Biomarker Score was then used to verify the possibility to distinguish fertile controls from infertile patients. The mean value of the Biomarker Score of fertile controls was 2.01 ± 1.42, whilst infertile patients of the master and of the validation groups had values of 12.27 ± 3.15 and of 11.41 ± 4.09, respectively (Fig. 2A). The two groups of infertile males had similar mean values of the Biomarker Score that were 6.1 and 5.7 times higher, respectively, than that of controls ($P < 0.001$). The lack of differences between the master and the validation groups allowed pooling the data of the Biomarker Score into a single cohort of infertile males, for the further statistical analysis.

The distribution frequency of the Biomarker Score of fertile controls and pooled infertile patients is shown in Fig. 2B. About the 93% of controls had Biomarker Score values from 0 to 3, with the remaining 7% scoring 4. No one of the 48 controls had Biomarker Score values higher than 4. Conversely, pooled infertile patients had Biomarker Scores ranging from 5 to 21, with the 87.5% of them having values comprised between 9 and 16.

Categorization of pooled infertile patients showed that those without (N) and those with (ATO + A + T + O) spermiogram anomalies

Table II Values of water- and fat-soluble low-molecular weight compounds representative of antioxidant defences, oxidative/nitrosative stress, purines, pyrimidines and energy metabolism determined by HPLC in seminal plasma of control fertile males.

Compound	Fertile controls ($n = 48$)	Reference intervals (5–95% percentiles)
GSH	17.64 ± 4.12	10.00–26.00
Ascorbic acid	286.01 ± 75.29	140.00–440.00
All-trans retinoic acid	0.003 ± 0.001	0.001–0.005
All-trans retinol	0.075 ± 0.030	0.033–0.130
α-Tocopherol	2.65 ± 1.31	1.00–4.80
γ-Tocopherol	0.052 ± 0.024	0.027–0.110
Total carotenoids	0.233 ± 0.126	0.070–0.470
Coenzima Q_{10}	0.030 ± 0.021	0.014–0.063
MDA	0.005 ± 0.005	0.001–0.015
8-OHdG	N.D.	N.D.
Nitrites	2.76 ± 0.85	1.30–3.95
Nitrates	22.63 ± 13.55	9.00–57.00
Creatinine	144.01 ± 52.70	70.00–260.00
Cytosine	1.50 ± 0.85	0.25–2.50
Cytidine	2.00 ± 0.82	0.70–3.50
Uracil	3.67 ± 1.52	1.10–7.00
β-Pseudouridine	3.51 ± 1.36	1.40–7.00
Hypoxanthine	6.38 ± 2.74	2.50–11.00
Xanthine	39.08 ± 9.47	20.00–60.00
Uridine	2764.57 ± 766.04	1750.00–3400.00
Uric acid	222.37 ± 44.13	135.00–325.00
Inosine	3.04 ± 0.62	1.30–4.60
Guanine	63.79 ± 15.31	30.00–85.00
Guanosine	69.46 ± 22.52	30.00–95.00
Orotic acid	43.81 ± 9.67	25.00–65.00
Adenosine	43.99 ± 10.03	25.00–70.00

Values of controls are the mean ± S.D. and, including values of reference intervals, are expressed as μmol/l seminal plasma. GSH = reduced glutathione; total carotenoids = sum of astaxanthin + lutein + zeaxanthin + *trans*-β-*apo*–8′-carotenal + β-cryptoxanthin + lycopene + α-carotene + β-carotene; MDA = malondialdehyde; 8-OHdG = 8-hydroxy-2′-deoxyguanosine; N.D. = not detectable. Sample processing and HPLC conditions allowing the separation and quantification of the compounds under evaluation are described under Materials and Methods.

Table III Values of water- and fat-soluble low-molecular weight compounds representative of antioxidant defences, oxidative/nitrosative stress, purines, pyrimidines and energy metabolism determined by HPLC in seminal plasma of control fertile males and in the master group of infertile males.

Compound	Fertile controls ($n = 48$)	Infertile patients (master group, $n = 96$)
GSH	17.64 ± 4.12	16.23 ± 10.11
Ascorbic acid	286.01 ± 75.29	197.44 ± 139.09[a]
All-trans retinoic acid	0.003 ± 0.001	0.001 ± 0.001[a]
All-trans retinol	0.075 ± 0.030	0.037 ± 0.026[a]
α-Tocopherol	2.65 ± 1.31	1.00 ± 0.65[a]
γ-Tocopherol	0.052 ± 0.024	0.027 ± 0.019[a]
Total carotenoids	0.233 ± 0.126	0.047 ± 0.035[a]
Coenzima Q_{10}	0.030 ± 0.021	0.029 ± 0.016
MDA	0.005 ± 0.005	0.213 ± 0.104[a]
8-OHdG	N.D.	0.093 ± 0.056[a]
Nitrites	2.76 ± 0.85	9.66 ± 8.32[a]
Nitrates	22.63 ± 13.55	36.16 ± 28.52[a]
Creatinine	144.01 ± 52.70	267.20 ± 132.07[a]
Cytosine	1.50 ± 0.85	2.64 ± 1.89[a]
Cytidine	2.00 ± 0.82	6.17 ± 5.87[a]
Uracil	3.67 ± 1.52	6.86 ± 4.53[a]
β-Pseudouridine	3.51 ± 1.36	7.39 ± 4.54[a]
Hypoxanthine	6.38 ± 2.74	13.83 ± 8.69[a]
Xanthine	39.08 ± 9.47	62.15 ± 34.52[a]
Uridine	2764.57 ± 766.04	1806.38 ± 843.33[a]
Uric acid	222.37 ± 44.13	229.14 ± 99.31
Inosine	3.04 ± 0.62	4.82 ± 4.23[a]
Guanine	63.79 ± 15.31	30.08 ± 17.06[a]
Guanosine	69.46 ± 22.52	34.72 ± 27.12[a]
Orotic acid	43.81 ± 9.67	42.54 ± 21.71
Adenosine	43.99 ± 10.03	40.43 ± 17.66

Values are the mean ± S.D. and are expressed as μmol/l seminal plasma. Values of patients were calculated without categorizing them according to the anomalies on the spermiogram. GSH = reduced glutathione; total carotenoids = sum of astaxanthin + lutein + zeaxanthin + *trans*-β-*apo*–8′-carotenal + β-cryptoxanthin + lycopene + α-carotene + β-carotene; MDA = malondialdehyde; 8-OHdG = 8-hydroxy-2′-deoxyguanosine; N.D. = not detectable. Sample processing and HPLC conditions allowing the separation and quantification of the compounds under evaluation are described under Materials and Methods.
[a]Significantly different compared to controls, $P < 0.001$.

had mean values of the Biomarker Score of 10.88 ± 2.53 ($P < 0.001$ compared to controls) and 13.09 ± 2.11 ($P < 0.001$ compared to controls; $P < 0.05$ compared to infertile N), respectively, indicating a more compromised metabolic profile of seminal plasma when spermiogram anomalies are present (Fig. 3A).

The distribution frequency (Fig. 3B) evidenced that the Biomarker Score values tend to cluster infertile N and pooled infertile ATO + A + T + O into two overlapping but distinct groups, with the former being more frequently distributed within Biomarker Score ranging from 8 to 12 and the latter within Biomarker Score ranging from 12 to 17.

In the pooled cohort of infertile males of the master and the validation groups, the Biomarker Score was also applied to discriminate infertile patients without (N) from those with (ATO + A + T + O) anomalies in the spermiogram. Based on the Biomarker Score number and using the Bayes theorem (Salehi *et al.*, 2007), the posterior probabilities for each patient to belong to a category of fertile controls or infertile males without (N) or with spermiogram anomalies (ATO + A + T + O) were calculated (Fig. 4). From these three curves, it is evident that a Biomarker Score ≤4 corresponds to metabolic patterns of seminal plasma found in fertile control only, i.e. fertile and infertile males are clustered into two distinct, not superimposable groups of Biomarker Score. Values of the Biomarker Score from 5 to 10 are

Table IV Values of water- and fat-soluble low-molecular weight compounds representative of antioxidant defences, oxidative/nitrosative stress, purines, pyrimidines and energy metabolism determined by HPLC in seminal plasma of control fertile males and in the master and validation groups of infertile males. No differences were recorded in any biochemical parameter of seminal fluid between the two groups of infertile patients.

Compound	Controls ($n = 48$)	Infertile patients (master group, $n = 96$)	Infertile patients (validation group, $n = 44$)
GSH	17.64 ± 4.12	16.23 ± 10.11	15.16 ± 8.26
Ascorbic acid	286.01 ± 75.29	197.44 ± 139.09[a]	162.65 ± 115.11[a]
All-trans retinoic acid	0.003 ± 0.001	0.001 ± 0.001[a]	0.001 ± 0.001[a]
All-trans retinol	0.075 ± 0.030	0.037 ± 0.026[a]	0.042 ± 0.016[a]
α-Tocopherol	2.65 ± 1.31	1.00 ± 0.65[a]	0.88 ± 0.34[a]
γ-Tocopherol	0.052 ± 0.024	0.027 ± 0.019[a]	0.035 ± 0.024[a]
Total carotenoids	0.233 ± 0.126	0.047 ± 0.035[a]	0.071 ± 0.053[a]
Coenzima Q_{10}	0.030 ± 0.021	0.029 ± 0.016	0.026 ± 0.021
MDA	0.005 ± 0.005	0.213 ± 0.104[a]	0.183 ± 0.099[a]
8-OHdG	N.D.	0.093 ± 0.056[a]	0.112 ± 0.75[a]
Nitrites	2.76 ± 0.85	9.66 ± 8.32[a]	14.51 ± 11.44[a]
Nitrates	22.63 ± 13.55	36.16 ± 28.52[a]	41.09 ± 24.67[a]
Creatinine	144.01 ± 52.70	267.20 ± 132.07[a]	292.02 ± 146.45[a]
Cytosine	1.50 ± 0.85	2.64 ± 1.89[a]	4.03 ± 2.85[a]
Cytidine	2.00 ± 0.82	6.17 ± 5.87[a]	8.33 ± 6.91[a]
Uracil	3.67 ± 1.52	6.86 ± 4.53[a]	6.01 ± 3.68[a]
β-Pseudouridine	3.51 ± 1.36	7.39 ± 4.54[a]	7.03 ± 2.12[a]
Hypoxanthine	6.38 ± 2.74	13.83 ± 8.69[a]	16.38 ± 9.84[a]
Xanthine	39.08 ± 9.47	62.15 ± 34.52[a]	68.23 ± 29.62[a]
Uridine	2764.57 ± 766.04	1806.38 ± 843.33[a]	1654.70 ± 903.15[a]
Uric acid	222.37 ± 44.13	229.14 ± 99.31	206.41 ± 87.13
Inosine	3.04 ± 0.62	4.82 ± 4.23[a]	5.77 ± 3.23[a]
Guanine	63.79 ± 15.31	30.08 ± 17.06[a]	25.69 ± 7.06[a]
Guanosine	69.46 ± 22.52	34.72 ± 27.12[a]	31.47 ± 12.19[a]
Orotic acid	43.81 ± 9.67	42.54 ± 21.71	54.26 ± 19.30
Adenosine	43.99 ± 10.03	40.43 ± 17.66	48.06 ± 16.49

Values are the mean ± S.D. and are expressed as μmol/l seminal plasma. GSH = reduced glutathione; total carotenoids = sum of astaxanthin + lutein + zeaxanthin + *trans*-β-*apo*−8′-carotenal + β-cryptoxanthin + lycopene + α-carotene + β-carotene; MDA = malondialdehyde; 8-OHdG = 8-hydroxy-2′-deoxyguanosine; N.D. = not detectable. Sample processing and HPLC conditions allowing the separation and quantification of the compounds under evaluation are described under Materials and Methods.
[a]Significantly different compared to controls, $P < 0.001$.

more probably associated with infertile N, whilst those ranging from 14 to 21 are more probably found in infertile ATO + A + T + O patients.

Discussion

Data reported in the present study demonstrate that the biochemical analysis of seminal plasma, targeted to quantify compounds related to redox energy state, antioxidant defences, oxidative/nitrosative stress, purines, pyrimidines and energy metabolism, might be a useful laboratory tool in the diagnostic evaluation of male infertility. Thanks to this 'Redox Energy Test', measuring 26 water- and fat-soluble low-molecular weight compounds, we found that 21/26 of them are significantly different in infertile patients in comparison with values determined in fertile controls, therefore assuming the biochemical meaning of biomarkers of male infertility. The results were obtained in a master group and confirmed in an independent validation group of infertile males. The exact quantification of the different compounds was performed using two validated HPLC methods characterized by reliability, sensitivity, linearity, reproducibility and recovery previously described in detail elsewhere (Tavazzi *et al.*, 2005; Lazzarino *et al.*, 2017a).

Results of the present study suggest that infertility seems to cause a significant decrease of antioxidants in seminal plasma. Particularly, in infertile males of both the master and the validation group mean values of ascorbic acid (representing the most abundant antioxidant of seminal plasma) were ~1.6 times lower than those measured in fertile controls ($P < 0.001$). All fat-soluble antioxidants (*all*-trans retinoic acid, *all*-trans retinol, α-tocopherol, γ-tocopherol, total carotenoids), but coenzyme Q_{10}, were significantly lower in males with infertility than in healthy fertile subjects ($P < 0.001$). It should be recalled that the analyses currently

Table V Values of the 21 water- and fat-soluble low-molecular weight biomarkers of male infertility representative of antioxidant defences, oxidative/nitrosative stress, purines, pyrimidines and energy metabolism, determined by HPLC in seminal plasma of control fertile and both groups of infertile males (master and validation groups) categorized according to the anomalies in the spermiogram.

Compound	Controls ($n = 48$)	Infertile N ($n = 42$)	Infertile ATO + A + T + O ($n = 98$)
GSH	17.64 ± 4.12	18.04 ± 8.81	15.98 ± 7.77
Ascorbic acid	286.01 ± 75.29	293.67 ± 101.51	131.70 ± 91.52[a,b]
All-trans retinoic acid	0.003 ± 0.001	0.001 ± 0.001[a]	0.001 ± 0.001[a]
All-trans retinol	0.075 ± 0.030	0.059 ± 0.037	0.030 ± 0.026[a,b]
α-Tocopherol	2.65 ± 1.31	1.52 ± 0.86[a]	0.881 ± 0.437[a,b]
γ-Tocopherol	0.052 ± 0.024	0.025 ± 0.020[a]	0.022 ± 0.017[a]
Total carotenoids	0.233 ± 0.126	0.073 ± 0.024[a]	0.040 ± 0.032[a,b]
Coenzima Q_{10}	0.030 ± 0.021	0.029 ± 0.016	0.027 ± 0.018
MDA	0.005 ± 0.005	0.086 ± 0.107[a]	0.229 ± 0.176[a,b]
8-OHdG	N.D.	0.056 ± 0.092[a]	0.139 ± 0.081[a,b]
Nitrites	2.76 ± 0.85	9.38 ± 7.14[a]	8.92 ± 6.43[a]
Nitrates	22.63 ± 13.55	31.73 ± 18.33	40.95 ± 24.02[a]
Creatinine	144.01 ± 52.70	216.84 ± 82.08[a]	252.58 ± 112.51[a]
Cytosine	1.50 ± 0.85	2.53 ± 1.18[a]	2.34 ± 2.00[a]
Cytidine	2.00 ± 0.82	4.65 ± 3.71[a]	8.11 ± 5.01[a,b]
Uracil	3.67 ± 1.52	5.46 ± 2.54[a]	5.35 ± 3.24[a]
β-Pseudouridine	3.51 ± 1.36	7.14 ± 4.55[a]	9.58 ± 5.14[a]
Hypoxanthine	6.38 ± 2.74	11.73 ± 8.96[a]	14.69 ± 9.36[a]
Xanthine	39.08 ± 9.47	51.72 ± 20.43[a]	65.47 ± 40.95[a]
Uridine	2764.57 ± 766.04	2365.70 ± 715.98	1702.33 ± 878.40[a,b]
Uric acid	222.37 ± 44.13	242.06 ± 88.31	254.11 ± 126.32
Inosine	3.04 ± 0.62	3.45 ± 2.77	6.50 ± 4.32[a,b]
Guanine	63.79 ± 15.31	41.20 ± 14.97[a]	27.66 ± 17.84[a,b]
Guanosine	69.46 ± 22.52	42.81 ± 24.90[a]	32.09 ± 22.18[a,b]
Orotic acid	43.81 ± 9.67	46.36 ± 18.35	39.76 ± 22.16
Adenosine	43.99 ± 10.03	48.70 ± 16.15	43.27 ± 16.43

Values are the mean ± S.D. and are expressed as μmol/l seminal plasma. Patients were categorized according to the spermiogram into N = normozoospermic and ATO + A + O + T = asthenoteratooligozoospermic + asthenozoospermic + teratozoospermic + oligozoospermic. GSH = reduced glutathione; Total carotenoids = sum of astaxanthin + lutein + zeaxanthin + *trans*-β-*apo*−8′-carotenal + β-cryptoxanthin + lycopene + α-carotene + β-carotene; MDA = malondialdehyde; N.D. = not detectable. Sample processing and HPLC conditions allowing the separation and quantification of the compounds under evaluation are described under Materials and Methods.

[a]Significantly different from controls, $P < 0.001$.

[b]Significantly different from normozoospermic, $P < 0.01$.

in use for a gross evaluation of certain pathological phenomena (ROS damage and production, decrease of total antioxidant capacity) does not qualitatively and quantitatively measure the different antioxidants, nor to evaluate nitrosative stress, nor to determine eventual signs of energy imbalance (Pahune *et al.*, 2013; Agarwal *et al.*, 2015; Homa *et al.*, 2015; Roychoudhury *et al.*, 2016).

With respect to previous studies (Mehraban *et al.*, 2005; Kiziler *et al.*, 2007), we here found that MDA in seminal plasma of fertile controls was barely detectable (0.005 ± 0.005 μmol/l). Conversely, 40 times higher MDA values were detected in the master and the validation groups of infertile males (0.213 ± 0.104 and 0.183 ± 0.099 μmol/l, respectively; $P < 0.0001$ compared to fertile controls). Increased concentrations of nitrites and nitrates, considered as stable end products of nitric oxide metabolism (Csonka *et al.*, 2015), allowed to hypothesize that, under infertility conditions, the highly damaging peroxynitrite might be formed (Ramdial *et al.*, 2017). The detection of 8-OHdG in infertile males seems to corroborate this hypothesis.

Infertility seems to be also characterized by an increased rate in the degradation pathway of adenine nucleotides, usually occurring during imbalance between ATP production and consumption (Domański *et al.*, 2007; Bracko *et al.*, 2014; Amorini *et al.*, 2016; Lazzarino *et al.*, 2017b). Changes in pyrimidine metabolism were also evident in infertile males of both groups, who showed remarkable seminal plasma decrease in uridine with consequent increase in its ribose-free catabolite uracil. Uridine and uridine derivatives are involved in a variety of important biochemical functions (Maher *et al.*, 2008; Bond and

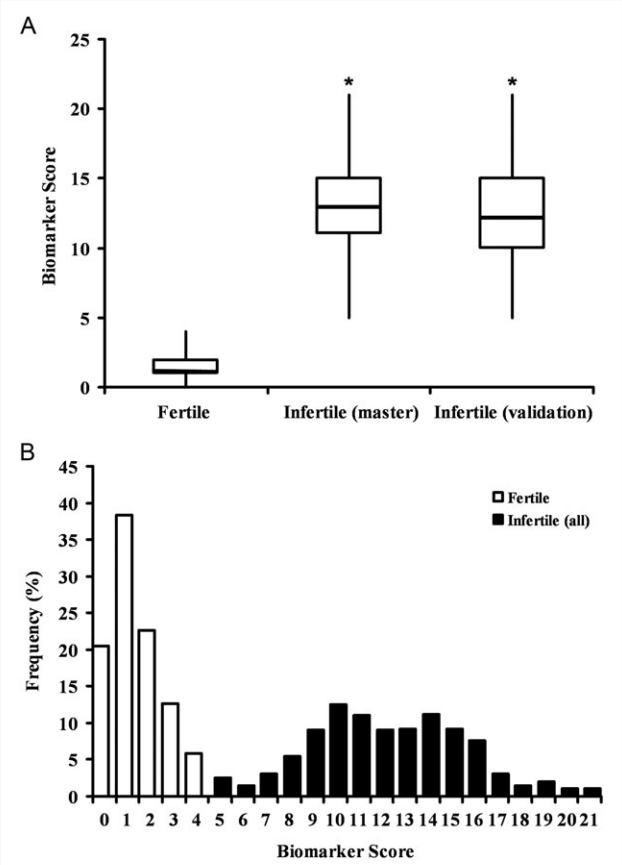

Figure 2 Box plot (**A**) reporting the values of the Biomarker Score obtained in fertile controls (n = 48) and in the two independent groups (master, n = 96; validation, n = 44) of infertile patients. Since no difference was recorded when comparing Biomarker Score values of the two groups, patients were combined into a single cumulative cohort of infertile males. Distribution frequency (**B**) of the Biomarker Score in fertile controls and in the cumulative cohort of infertile patients. About the 94% of controls had Biomarker Score values from 0 to 3, with the remaining 6% scoring 4 (none of the controls had values higher than 4). Conversely, pooled infertile patients had Biomarker Scores ranging from 5 to 21, with more than 90% of them ranging between 8 and 16. *Significantly different from controls, P < 0.001.

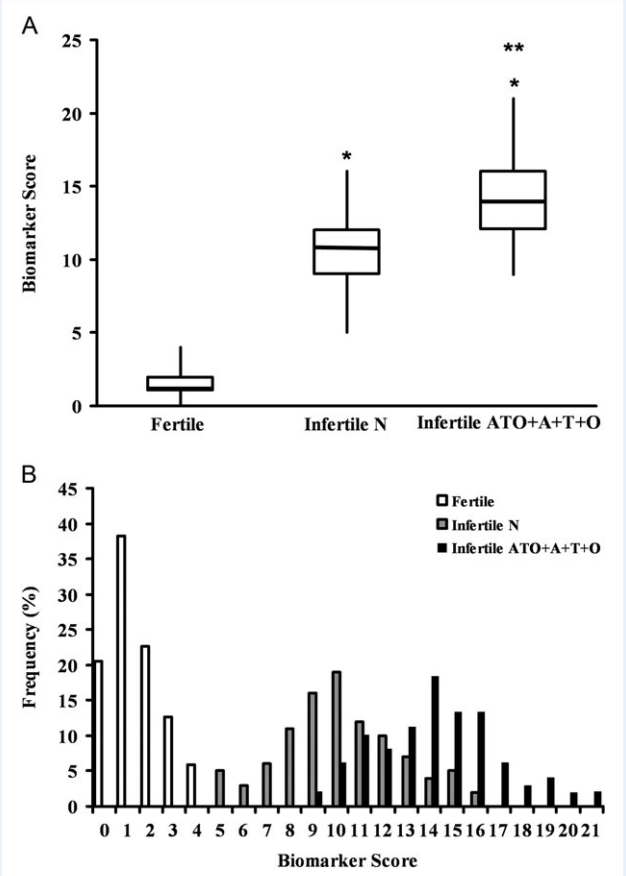

Figure 3 Box plot (**A**) reporting values of the Biomarker Score obtained in fertile controls and in the cumulative cohort of infertile patients categorized into normozoospermic (N, n = 42) and astheno-teratooligozoospermic + astheno + terato + oligozoospermic (ATO + A + T + O, n = 98). Distribution frequency (**B**) of the Biomarker Score recorded in fertile controls and in the groups of patients categorized into those without (N) and with (ATO + A + T + O) anomalies in the spermiogram. *Significantly different from fertile controls, P < 0.001. **Significantly different from infertile N, P < 0.01.

Hanover, 2015). Hence, the decrease in seminal plasma uridine might affect several biochemical processes of spermatozoa, with negative consequences on male fertility.

When infertile patients of the master and the validation groups were first pooled into a single cohort and then categorized into those without (N) and with (ATO + A + T + O) alterations in the spermiogram, partial differences in their profiles of seminal plasma metabolites (ascorbic acid, *all-trans* retinol, α-tocopherol, total carotenoids MDA, 8-OHdG, cytidine, uridine, inosine, guanine, guanosine), were observed.

Introducing the Biomarker Score as an index of the metabolic profile, cumulatively representing the number of biochemical anomalies of human seminal plasma, we found that its values in fertile controls ranged between 0 and 4, whilst those in infertile patients of both groups ranged between 5 and 21. Hence, the Biomarker Score allowed to cluster fertile and infertile males into two clearly distinguishable groups. In other words, it seems that fertility tolerates a maximum of 4/21 abnormal values among the biomarkers identified in human seminal plasma. The further application of the Biomarker Score to grouped infertile patients, categorized into infertile N and infertile ATO + A + T + O, permitted to evidence that infertile ATO + A + T + O had higher mean values of the Biomarker Score (P < 0.005 compared to infertile N). Additionally, it was also possible to show that the frequency of distribution of their Biomarker Score values appeared different from that of infertile N. The posterior probability curves of fertile controls, infertile N and infertile ATO + A + T + O suggest that the Biomarker Score clearly distinguished fertile controls from the two other groups of infertile patients and, for certain values of this index, infertile N from infertile ATO + A + T + O. It is of particular relevance to underline that infertile N, i.e. the category of idiopathic patients

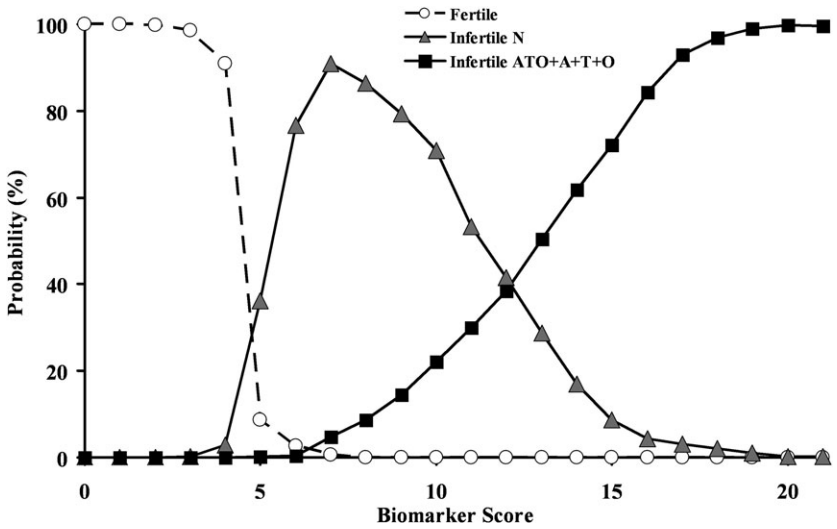

Figure 4 Posterior probability curves of Biomarker Score determined in fertile controls and in the groups of patients categorized into those without (N) and with (ATO + A + T + O) anomalies in the spermiogram. The curves were calculated as described in Materials and Methods (statistical analyses).

with normal spermiogram, had Biomarker Score values higher than those of fertile controls, indicating that biochemical/metabolic alterations are possibly involved in their inability to reproduce.

We found striking differences between the metabolic profiles of seminal plasma and serum from peripheral blood (Ronquist and Niklasson, 1984; Vural *et al.*, 1999; Tavazzi *et al.*, 2005; Wang *et al.*, 2013). Ascorbic acid, uridine, guanosine, guanine, xanthine, inosine and adenosine were ~4.5, 500, >10 000, >10 000, 25, 25 and 25 times higher, respectively, in seminal plasma than in circulating serum, clearly evidencing that these two fluids have different biochemical profiles of various low-molecular weight metabolites. Since ascorbic acid in human tissues is of exclusive dietary origin, the existence of to date unknown transport mechanisms from serum to seminal plasma against a concentration gradient, ultimately allowing vitamin C accumulation in seminal plasma, should be operative. The concentrations of selected purines and pyrimidines in seminal plasma also cast questions about: (i) the biological meaning and the potential active role of these metabolites during fertilization (Bellezza and Minelli, 2017) and (ii) the mechanisms (and the energy expenditure) allowing their accumulation in seminal plasma.

In conclusion, we evidenced that the 'Redox Energy Test', by measuring selected compounds representative of antioxidant defences, oxidative/nitrosative stress, purines, pyrimidines and energy metabolism, was capable to highlight a set of 21 compounds acting as biomarkers of male infertility. Metabolic profiles of human seminal plasma were cumulated into the Biomarker Score associating the number of biochemical/metabolic anomalies with infertility. Further studies to reinforce the clinical meaning of the 'Redox Energy Test' (by increasing the number of both fertile controls and infertile patients), as well as to determine the efficacy of specific treatments on the biochemical quality of seminal plasma and, more important, on the capacity of fertilization, are in progress.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

G.L. contributed to sample preparation for HPLC analysis, carried out the HPLC determination of fat-soluble compounds and wrote in part the article. I.L. carried out spermiogram, contributed to sample preparation for HPLC analysis and revised the article. L.M. carried out spermiogram, performed collection of clinical data and revised the article. A.M.A. carried out the HPLC determination of water-soluble compounds and revised the article. S.L. evaluated results of the HPLC analyses and revised the article. E.D.S. carried out the statistical tests and the introduction of the Biomarker Score and revised the article. G.C. evaluated results of the HPLC analyses, carried out basic statistic evaluation of the data and revised the article. S.D.'U. evaluated results of the HPLC analyses and revised the article. G.P. performed the clinical evaluation of the patients of the validation group and revised the article. G.L. analysed results and wrote the article. B.T. analysed results and wrote the article. P.B. performed the clinical evaluation of the patients of the master group and revised the article.

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Conflict of interest

Authors have no conflicts of interest to declare.

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