SELECTED COMPOUNDS REPRESENTATIVE OF THE BIOCHEMICAL QUALITY OF HUMAN SEMINAL PLASMA: A USEFUL LABORATORY TOOL TO EVALUATE MALE INFERTILITY

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Running title: Biochemical quality of seminal plasma in infertile males

ABSTRACT

Study question: Is the determination of antioxidants, oxidative/nitrosative stress-related compounds, purines, pyrimidines, energy-related metabolites in human seminal plasma a useful laboratory in the diagnosis of male infertility?

Summary answer: The determination of 26 metabolites in seminal plasma allowed to calculate a cumulative index, named Biomarker Score, that discriminates fertile controls from infertile patients and differentiate various types of infertility.

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

Study design, size, duration: Seminal plasma of 38 fertile controls and 96 infertile patients, categorized on the spermiogram into normozoospermic (N), asthenoteratooligozoospermic (ATO), asthenozoospermic (A), teratozoospermic (T), oligozoospermic (O), were collected from September 2016 to December 2017. Samples were analysed in blind to determine various low molecular weight compounds of different biochemical meaning, with the aim of finding metabolite profiles related to male infertility.

Participants/materials, setting, methods: In all seminal plasma, 26 water- and fat-soluble compounds were analyzed using sensitive, reproducible, low-cost high-performance liquid chromatographic methods. According to the results of the spermiogram, infertile patients were assigned to the subgroups of normozoospermic (N, n = 28), asthenoteratooligozoospermic (ATO, n

= 31) or astheno + terato + oligozoospermic (A + T + O, n = 37). The subgroups ATO and A + T + O were pooled into a single subgroup (n = 68) for the subsequent statistical comparison.

Main results and the role of chance: Results of this Redox Energy Test indicated that 21/26 compounds assayed discriminated fertile from pooled infertile subjects, gaining the meaning of male infertility biomarkers. Infertile patients without (N) or with (pooled ATO + A + T + O) spermiogram anomalies differed in some biomarkers (ascorbic acid, all-trans retinol, α-tocopherol, cytidine, uridine, guanine). Using an index cumulating the different biochemical seminal plasma anomalies (Biomarker Score), we found that fertile controls, infertile N, pooled infertile ATO + A + T + O had mean Biomarker Scores of 1.47 ± 1.23, 11.15 ± 2.84 and 13.46 ± 2.87, respectively, ($p < 0.001$ and $p < 0.01$ compared to controls or infertile N). These differences were reinforced by distribution frequencies and posterior probability curves of the Biomarker Score in the three groups.

Limitations, reasons for caution: Results of the study were obtained in relatively limited number of human seminal plasma samples. Although this did not hinder to discriminate fertile controls from infertile males, using the Redox Energy Test and the Biomarker Score, it did not allow to evaluate their correlation with the different anomalies of the spermiogram.

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, might be considered a new clinically useful analytical approach highly recommendable to monitor the effectiveness of specific therapeutic strategies.

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KEY WORDS: Male infertility; human seminal plasma; oxidative/nitrosative stress; mitochondrial dysfunction; biochemical analyses.

INTRODUCTION

Infertility, defined as the inability to achieve pregnancy after twelve months of regular intercourse, is a multi-factorial 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).

According to the World Health Organization (WHO) guidelines, the male factor is considered to be involved when one or more semen parameters (sperm concentration, motility and morphology) are abnormal. Epidemiological studies have indicated that a male factor is involved in about the 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; Inters and Walsh, 2014; Szkodziak et al., 2016).

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: extra-testicular (alterations of sperm transport, ejaculation disturbances, inability to perform sexual intercourse), testicular (congenital or acquired diseases, such as varicocele or inflammation), pre-testicular (disorders of the neuroendocrine system of different origin) and idiopathic (i.e. for those in whom the primary cause is not clear defined) (Bhasin, 2007; Pfeifer et al., 2013; Ko et al., 2014; Inters and Walsh, 2014; Szkodziak et al., 2016; Cui et al., 2016). It is worth underlining that this last group accounts for the 30-40% of cases of male infertility.

The latest updates of WHO guidelines (World Health Organization, 2010), besides describing the correct procedures for the morpho-functional evaluation of the sperm through the spermiogram, reserved a chapter to optional procedures, defined as “not necessary for routine semen analysis, but that may be useful in certain circumstances for “diagnostic or research purposes”. These include the evaluation of oxidative stress (Homa et al., 2015).

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 (Lanzafame et al., 2009; Agarwal et al. 2014a; Ko et al., 2014). Notwithstanding, physiological ROS levels play an important role in proper sperm processes such as spermatozoa maturation, (Aitken et al., 2004; Baker and Aitken, 2004), capacitation (Agarwal et al., 2014b), hyperactivation (de Lamirande and Cagnon, 1993; Suarez, 2008), acrosome reaction (Breitbart and Naor, 1999) and sperm-oocyte fusion (Aitken et al., 1995). It is however well demonstrated that the excess of these harmful compounds induce a condition of oxidative stress triggering irreversible modification to biologically fundamental molecules of spermatozoa, such as peroxidation of membrane phospholipids (Aitken et al., 2016), oxidative modification to structure and function of proteins (O'Flaherty and Matsushita-Fournier, 2017), oxidative damages to DNA (Bisht and Dada, 2017). Increased ROS production may originate either from intra- or extra-spermatozoa sources. In the first case, dysfunctional mitochondria are the main site of ROS production through an incomplete reduction of molecular oxygen caused by malfunctioning of the electron transfer chain and generating superoxide anions (Cassina et al., 2015). Mitochondrial malfunctioning causes a decreased capacity to generate ATP through oxidative phosphorylation coupled to electron transfer chain, with a consequent cell energy deficit (Cassina et al., 2015), thereby linking redox and energy metabolism. In the second case, activated leukocytes resulting from inflammation and infection are significant intrinsic producers of ROS in semen. The increase of leukocytes in the semen may even be the result of environmental factors, long sexual abstinence, or varicocele (Wolff, 1995; Walczak-Jedrzejowska et al., 2013). Immature spermatozoa with abnormal head morphology and cytoplasmic retention, as well as damaged, deficient or abnormal spermatozoa as a result of impaired spermatogenesis are an additional important source of increased ROS levels in semen (Agarwal et al., 2014c).

In various biological context, it has been demonstrated that oxidative stress is associated with nitrosative stress (Kanaan and Harper, 2017), caused by an excess production of Reactive Nitrogen

Species (RNS). The coincidence of these phenomena, known as oxidative/nitrosative stress, is known to generate peroxynitrite ($ONOO^{\cdot}$), one of the most dangerous free radical molecule for the biological systems (Ramdial et al., 2017).

Any cell type, including spermatozoa, is well equipped to scavenge some specific ROS with highly efficient enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase. However, the most damaging ROS and RNS, hydroxyl radical and peroxynitrite, respectively, are scavenged through the intervention of low-molecular weight antioxidants such as ascorbic acid, uric acid, reduced glutathione (Regoli and Winston, 1999). Even semen quality is probably strictly dependent on the level of hydrophilic (ascorbic acid, uric acid, reduced glutathione) and hydrophobic (vitamin E, vitamin A, carotenoids, coenzyme Q_{10}) low molecular weight antioxidants in seminal plasma (Ghyasvand et al., 2015). The concentration of most of these compounds in body fluids, including seminal plasma, is strictly dependent on the diet regimen and represents (also for semen) a fundamental defense system towards ROS toxicity (Fang et al., 2002).

Notwithstanding the attention given to the connection between ROS and male infertility, and to the connection between ROS damages and antioxidant defence systems, to date the concentrations of the full pattern of low molecular weight antioxidants in seminal plasma of fertile and infertile males are, surprisingly, still unknown. Referring to hydrophobic antioxidants, we have very recently determined the concentration of vitamin E, vitamin A, carotenoids and coenzyme Q_{10} in seminal plasma of fertile male finding a peculiar profile of this biological fluid (Lazzarino et al., 2017). According to what aforementioned, it seems clear the importance of performing an accurate analysis of redox and energy state-related metabolites allowing the quality evaluation of human seminal plasma, with the aim to find new biochemical biomarkers related to male infertility.

In this study, we carried out the analysis of hydrophilic and hydrophobic antioxidants (named *Redox Energy Test*), of metabolites related to energy metabolism, of compounds related to oxidative/nitrosative stress (a total of 26 compounds assayed) in two cohorts of fertile control subjects and infertile patients with different anomalies on the spermiogram. The determination of

this panel of metabolites in seminal plasma allowed to evidence striking differences between fertile and infertile males, as well as to calculate a Biomarker Score correlating with anomalies on the spermiogram.

MATERIALS AND METHODS

Patient population

The study was conducted according to the Declaration of Helsinki for Medical Research involving Human Subjects. Informed written consents were obtained from each control and patient enrolled in this study.

Patients (n = 96) with history of abnormal semen parameters and/or patients with a history of repeated in vitro fertilization/embryo transfer failure were recruited at the Alma Res Fertility Centre (Rome, Italy) from September 2016 to December 2017. They were clinically assessed according to the WHO guidelines (World Health Organization, 2000). Infections, varicocele, criptorchidism, chromosomic aberrations, obstructions, testicular tumors, and any systemic disease at the time of semen donation, were considered as exclusion criteria.

A group of fertile healthy volunteers (n = 38) was used as the control group. Normal semen parameters and proved fertility were used as the only inclusion criteria.

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 minutes, semen samples were observed in order to determine sperm motility, concentration and morphology according to WHO guidelines (World Health Organization, 2010).

Immediately after semen analysis, the complete freshly and liquefied semen samples were centrifuged for 15 minutes at 1860 *g*, in order to remove spermatozoa and other cell from seminal plasma. The seminal plasma was immediately withdrawn and processed for the HPLC analysis of selected low molecular weight metabolites. These biochemical analyses where carried out in blind.

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

Each seminal plasma sample was divided into two aliquots of 300 µl each. One aliquot was properly processed in order to ensure removal of protein and full recovery of an organic solvent extract containing hydrophobic low molecular weight compounds, including fat-soluble vitamins and antioxidants. The other aliquot, was properly processed to ensure protein removal and full recovery of a water extract containing hydrophilic low molecular weight compounds.

To obtain the organic solvent extracts, seminal plasma were processed according to a procedure recently described in detail elsewhere (Lazzarino et al., 2017). Briefly, one volume of seminal plasma sample (300 µl) was added with two volumes (600 µl) of HPLC-grade CH_3CN (Carlo Erba, Milan, Italy). After vigorous vortexing for 60 s, samples were incubated at 37◦C for 1 h in a water bath under agitation (to allow full extraction of fat-soluble compounds) and then centrifuged at 20,690 x g for 15 min at 4 C° to precipitate proteins. Clear supernatants were directly injected onto the HPLC column for the analysis of fat-soluble vitamins and antioxidants (Lazzarino et al., 2017).

To obtain the water extract, one volume of seminal plasma sample (300 µl) was added with two volumes (600 µl) of HPLC-grade CH_3CN. After vigorous vortexing for 60 s, samples were centrifuged at 20,690 x g for 15 min at 4 C° to precipitate proteins. Supernatants were then washed with 1.5 ml of chloroform (HPLC-grade), agitated vigorously for 90 seconds and centrifuged at 21,690 x g for 15 minutes at 4 C°. After two washings, the upper aqueous phase containing hydrophilic low molecular weight compounds (Tavazzi et al., 2005), and free of organic solvents, was directly injected onto the HPLC column for the analysis of purines, pyrimidines, antioxidants, compounds representative of oxidative/nitrosative stress, creatinine (Tavazzi et al., 2005).

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 by a recently described reversed phase HPLC (Lazzarino et al., 2017). Chromatographic separation was performed 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).

In the aqueous extract of deproteinized seminal fluid, water-soluble antioxidants (reduced glutathione (GSH) and ascorbic acid), biomarkers of oxidative/nitrosative stress (malondialdehyde, 8-hydroxy-2' -deoxyguanosine (8-OHdG), nitrite and nitrate), purines (hypoxanthine, xanthine, uric acid, inosine, adenosine, guanine and guanosine), pyimidine (uracil, β-pseudouridine, uridine, cytidine, cytosine and orotic acid) and creatinine (for a total of 20 compounds), were separated and quantified according to a well established isocratic ion-pairing HPLC method, formerly set up in our laboratory (Tavazzi et al., 2005) and used to measure similar pattern of metabolites in various biological samples (Bracko et al., 2014; Lazzarino et al., 2016; Amorini et al., 2016). Aqueous extracts were first diluted with doubly-distilled water (1:20, v/v) and loaded (200 µl) onto a Hypersil C-18, 250 × 4.6 mm, 5-µm particle size column, provided with its own guard column (ThermoElectron Italia, Rodano, Milan, Italy).

For both types of analyses the HPLC apparatus consisted of a Spectra System P4000 pump system (Thermo Fisher Scientific, Rodano, Milan, Italy), equipped with a highly-sensitive 5 cm light path flow cell, UV6000LP diode array detector (Thermo Fisher Scientific, Rodano, Milan, Italy), 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.

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 to those of runs of standard mixtures containing true compounds with known concentrations. It is worth underlining that, in the final calculations, the concentrations of astaxanthin, lutein, zeaxanthin, *trans*-β-apo-8'-carotenal, β-cryptoxanthin, lycopene, α-carotene, β-carotene were summed and this group of chemically homogenous compounds is reported hereinafter as total

252 carotenoids. Therefore, a final number of 26 metabolic parameters was took into consideration in
253 the subsequent statistical evaluations.
254
255 ***Statistical analysis***
256 Statistical analysis was performed by using the Statistical Package for Social Science (SPSS),
257 release 15.0. All data were first analyzed for normality of distribution using the Kolmogorov-
258 Smirnov test. Continuous variables were expressed as mean ± SD, categorical variables displayed
259 as frequencies and the appropriate parametric (Student‘s t-test or ANOVA) or non-parametric tests
260 (Mann-Whitney U-test, Kruskal-Wallis ANOVA, χ^2 test) were used to assess significance of the
261 differences between subgroups. A P value of less than 0.05 was considered statistically significant.
262 Within the panel of 26 different metabolites measured in seminal plasma, 21 fulfilled the
263 characteristic of being significantly different in infertile males, when compared to the values
264 recorded in fertile controls. For each of these 21 compounds we determined the 10° and 90°
265 percentile of its seminal plasma concentration in control subjects. Subsequently, the entire
266 population (fertile control and infertile patients) was stratified into two categories for each
267 biomarker: 1) Normal (0) = subjects with concentration ≥ 90° or ≤ 10° percentile of controls,
268 depending on biomarker value distributions in control subjects and infertile patients and 2) Positive
269 (1) = subjects with concentrations falling outside the 10° or 90° percentile of controls as just
270 described. Finally, each subject was associated to a specific biochemical pattern of markers
271 positivity and to a “Biomarker Score” (sum of the number of Positive categories), thus ranging from
272 0 (all biomarkers classified as “Normal”) to 21 (all biomarkers classified as “Positive”). The
273 schematic stepwise process to obtain the Biomarker Score, starting from the analysis of selected
274 compounds representative of antioxidant defences, of oxidative/nitrosative stress, of purine,
275 pyrimidine and energy metabolism, is reported in Fig. 1.
276 The Biomarker Score was then used to distinguish fertile controls from infertile patients, and then to
277 discriminate infertile N patients from infertile ATO patients and from infertile patients having any

other anomaly on the spermiogram (this groups was represented by the sum of the patients being A, T or O on the spermiogram).

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. Based on the Biomarker Score number and using the Bayes theorem (Salehi et al., 2007), these normal distributions allowed to calculate the posterior probabilities for each patient to belong to a specific category of spermiogram anomalies (N, ATO), or group of spermiogram anomalies (A + T + O).

RESULTS

Table I summarizes the clinical features of fertile controls and infertile patients. According to the results of the spermiogram, infertile patients were assigned to the subgroups of normozoospermic (N, n = 28), asthenoteratooligozoospermic (ATO, n = 31) or astheno + terato + oligozoospermic (A + T + O, n = 37). The subgroups ATO and A + T + O were pooled into a single subgroup (n = 68) for the subsequent statistical comparison. Therefore, according to the spermiogram, normozoospermic, patients could not be distinguished from controls in any of the morpho-functional evaluation of their respective semen samples, but still they were clinically infertile.

Evaluation of the biochemical quality of seminal plasma from fertile donors

Using a targeted metabolomic approach, aimed to assay selected compounds correlated to different biochemical functions, it was possible to evaluate initially the state of antioxidant defences, of oxidative/nitrosative stress, of purine, pyrimidine and energy metabolism of seminal plasma of control fertile subjects, and to determine their 5 - 95% percentiles. As shown in Table II, among all the water- and fat-soluble substances, ascorbic acid is the most abundant antioxidant with concentrations 4 to 6 times higher than those found in serum (Wang et al., 2013). Controls had minimal (MDA) or undetectable (8-OHdG) concentrations of compounds deriving from ROS-mediated damages to macromolecules (phospholipids of membranes and DNA, respectively), as well as low concentrations of nitrites and nitrates (considered as stable end products of nitric oxide production and metabolism) (Csonka et al., 2015). A peculiar pattern of purines, pyrimidines and energy-related metabolites distinguishes seminal plasma from other human body fluids, mainly serum. Particularly, uridine is 400 to 500 times more concentrated in seminal plasma (2540.57 ± 546.14 μmol/L) than in serum (4.86 ± 2.31 μmol/L) (Lazzarino et al., 2016), while guanine and guanosine are practically undetectable in serum and present in relevant amount in seminal plasma

(55.80 ± 14.48 and 65.17 ± 18.38 μmol/L, respectively). In general, all pyrimidines and purines, but uric acid, are more concentrated in seminal plasma than in serum.

The biochemical quality of seminal plasma in infertile males

The analysis of the metabolites of interest in seminal plasma of 96 males with infertility allowed to evidence a remarkably high number of anomalies. Table III reports the mean values of the 26 biochemical parameters found in seminal plasma of infertile patients independently from their categorization based on the results of the spermiogram. Impressively, 21/26 of the compounds measured in infertile males, 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 ($p < 0.01$). The aforementioned compounds therefore assumed the role of biomarkers for male infertility. Particularly evident were the decreases in ascorbic acid (-30%), α-tocopherol (-66%), *all*-trans retinol (-40%), total carotenoids (-80%), guanine (-48%), guanosine (-41%), and the increases in MDA (+4000%), 8-OHdG, nitrites (+270%), creatinine (+80%), cytosine (+140%), cytidine (+230%), hypoxanthine (+140%).

To verify whether the 21 biomarkers of the biochemical seminal plasma quality were differentially affected in infertile patients with diverse spermiogram categorizations, the cohort of males with infertility was divided into normozoospermic (N) and the sum of asthenoteratooligozoospermic (ATO) + asthenozoospermic (A) + teratozoospermic (T) + oligozoospermic (O), i.e. into the two unrefined groups of those infertile with no anomalies on the spermiogram and those infertile with anomalies on the spermiogram. Table IV summarizes the values of the 21 biomarkers found in seminal plasma of fertile controls, infertile N and infertile ATO + A + T + O. The very relevant result is that infertile N 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 who had no evident morpho-functional alterations according to the spermiogram. In addition to the aforementioned compounds, 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 of clinical utility to assess the biochemical quality of seminal plasma in infertile males

As previously mentioned, concentrations of the different compounds in seminal plasma of fertile subjects were initially used to calculate the 10° - 90° percentiles. Using these intervals relative to the compounds acting as biomarkers for infertility, each of the 21 metabolites of each patient scored 0 or 1. Subsequently, in both fertile and infertile males, a cumulative Biomarker Score was obtained as the sum of the metabolite scores (Fig. 1). These values ranged from 0 (each metabolite scoring 0) to 21 (each metabolite scoring 1).

Using this cumulative index representative of the biochemical quality of human seminal plasma, we initially calculated the Biomarker Scores in each fertile control (means ± S.D. = 1.47 ± 1.23) and infertile patient (means ± S.D. = 12.64 ± 3.02), i.e. infertile male donors (pooled cohort) had a mean Biomarker Score 8.6 times higher than that of controls ($p < 0.001$) (Fig. 2 A). The distribution frequency of the Biomarker Score of fertile controls and pooled infertile patients is shown in Fig. 2 B. About the 94% of controls had Biomarker Score values from 0 to 3, with the remaining 6% scoring 4. Conversely, pooled infertile patients had Biomarker Scores ranging from 5 to 21, with more than 89% of them being comprised between 9 and 16.

Additionally, infertile patients, categorized into those without (N) and those with (ATO + A + T + O) spermiogram anomalies (Fig. 3 A), had a mean Biomarker Score value of 11.15 ± 2.84 ($p < 0.001$ compared to controls) and 13.46 ± 2.87 ($p < 0.001$ compared to controls; $p < 0.01$ compared

363 to infertile N), respectively, indicating worse biochemical quality of seminal plasma when
364 spermiogram anomalies are present.
365 The distribution frequency (Fig. 3 B) allows to evidence that the values of the Biomarker Score tend
366 to cluster infertile N and pooled infertile ATO + A + T + O into two distinct groups, with the former
367 being more frequently distributed within Biomarker Score values ranging from 9 to 12 and the latter
368 within Biomarker Score values ranging from 13 to 16.
369 The probability to fell into controls, infertile N and pooled infertile ATO + A + T + O groups,
370 according to the Biomarker Score value and using the Bayes theorem, is shown in Fig. 4. From
371 these three curves, it is evident that a Biomarker Score ≤ 4 corresponds to seminal plasma metabolic
372 pattern found in fertile control only. Values of the Biomarker Score from 6 to 10 are more probably
373 associated with infertile N, whilst those ranging from 15 to 21 are more probably found in pooled
374 infertile ATO + A + T + O patients.
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376
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DISCUSSION

Data reported in the present study demonstrate that the biochemical analysis of seminal plasma, targeted to quantify compounds related to redox energy state, such as those connected to antioxidant defences, oxidative/nitrosative stress, purines, pyrimidines and energy metabolism, is a useful 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 were able to show, for the first time to the best of our knowledge, that 21/26 of them are significantly different in infertile patients in comparison with values determined in fertile controls, therefore assuming the biochemical and diagnostic meaning of biomarkers for male infertility.

The results obtained through the exact quantification of the compounds of interest demonstrate that seminal plasma of infertile males is generally characterized by a significant decrease of antioxidants. Particularly, in infertile males mean values of ascorbic acid, representing the most abundant antioxidant of seminal plasma, were about 1.4 times lower than those measured in fertile controls ($p < 0.001$), whilst no differences in the concentrations of GSH between the groups were recorded. 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 ($p < 0.001$). Previous in vitro experiments showed that astaxanthin (one of the lipid-soluble compounds incorporated into the total carotenoid value) improves sperm capacitation (Andrisani et al., 2015). This strongly corroborates the clinical relevance of the redox energy test performed in the present study as an essential tool for the subsequent targeted and differentiated treatments of infertile males

Referring to the antioxidant defences of seminal plasma, it is immediately evident the macroscopic discrepancy between the physiologic concentrations of the overall water- and the overall fat-soluble antioxidants (Table II), with the former (~520 μmol/L seminal plasma) being ~130 times more concentrated than the latter (~4 μmol/L seminal plasma). Compared to the concentrations of the

same compounds measurable in serum from peripheral blood under physiological conditions (~320 μmol/L serum) (Tavazzi et al., 2005; Vural et al., 1999), the overall water-soluble antioxidant concentration in serum is only ~8.5 times higher than its overall fat-soluble antioxidant concentration (~38 μmol/L serum) (Lazzarino et al., 2017).

Furthermore, among the different water-soluble antioxidants that we assayed in control seminal plasma (ascorbic acid, GSH and uric acid), ascorbic acid (~280 μmol/L seminal plasma) was largely the most abundant, with concentrations ~4.5 times higher than those detectable in serum from circulating blood (~60 μmol/L serum) (Tavazzi et al., 2005; Vural et al., 1999). Since ascorbic acid in human body is exclusively of dietary origin, this finding raises the question on the mechanisms underlying the transport from serum to seminal plasma against a concentration gradient, ultimately allowing vitamin C accumulation in human semen. The peculiar pattern of seminal plasma antioxidants (Tables II, III and IV) and the different biochemical and scavenging properties of ascorbic acid (Kojo, 2004), α-tocopherol (Grimm et al., 2016), *all*-trans-retinol (Wu et al., 2017), carotenoids (Galasso et al., 2017), with ascorbic acid and α-tocopherol acting in synergism and having more specific roles than other generic antioxidants (Kojo, 2004; Grimm et al., 2016), cast doubts on the very common clinical practice to administer antioxidants to infertile males without any prior analysis either in seminal plasma, or even in serum, aimed to properly measure the concentrations of each main antioxidant in this fluid. This hampers to evidence deficiency in one or more specific antioxidants, thus limiting the efficacy of this type of treatment and possibly performing administrations of unnecessary compounds.

As a consequence of the imbalance in specific antioxidants, seminal plasma of infertile males had clear molecular signatures of increased oxidative/nitrosative stress. With respect to previous studies, in which representative biomarkers of these phenomena were assayed using poorly specific and indirect methods (Mehraban et al., 2005; Kiziler et al., 2007), such as the questionable thiobarbituric acid assay for MDA (Tsikas et al., 2016) and the Griess assay for nitrites and nitrates (Romitelli et al., 2007), we here found that MDA in seminal plasma of fertile controls was barely

430 detectable (0.006 ± 0.009 μmol/L) but it was almost 42 times higher in pooled infertile males (0.249
431 ± 0.189 μmol/L; $p < 0.0001$). Increased concentrations of nitrites and nitrates (3.7 and 1.6 times,
432 respectively) allowed to evidence the simultaneous presence of oxidative and nitrosative stresses
433 and the actual possibility that, under infertility conditions, the highly damaging peroxynitrite might
434 be formed from the combination of nitric oxide NO and superoxide anions (Ramdial et al., 2017).
435 Our results were possible thanks to the HPLC methods we set up in the past that allows the direct
436 determination of true MDA (no derivatization required), as well as the simultaneous direct
437 measurement of nitrites and nitrates (no derivatization required). Using this technique we also
438 showed, for the first time to the best of our knowledge, that 8-OHdG, a well recognized index of
439 damage to DNA caused by ROS (Fenga et al., 2017), is detectable in seminal plasma of infertile
440 patients in a high percentage (Tables 3 an 4), suggesting that the imbalance in antioxidant defences
441 and increased oxidative/nitrosative stress might play a relevant role in the pathobiology of male
442 infertility.
443 Among the compounds of purine, pyrimidine and energy metabolism that we analyzed,
444 tremendously striking differences between seminal plasma and serum from peripheral blood were
445 found when comparing concentrations of uridine, guanosine, guanine, xanthine, inosine and
446 adenosine of the two fluids. Similarly to values reported in previous studies (Ronquist and
447 Niklasson, 1984), mean values of 2540.57 ± 546.14 μmol/L of uridine were detected in seminal
448 plasma from fertile donors, i.e. ~500 times higher than those (4.86 ± 2.31 μmol/L) measured in
449 serum from peripheral blood (Lazzarino et al., 2016). An even more striking difference occurred for
450 guanosine and guanine: in fact, whilst both compounds are nearly undetectable in serum samples
451 (Tavazzi et al., 2005), they both reached concentrations close to 60 μmol/L seminal plasma. Lastly,
452 inosine adenosine and xanthine were ~25 times more concentrated in seminal plasma than in serum
453 (Tavazzi et al., 2005). The composition in selected purine and pyrimidine compounds certainly
454 casts several questions about: i) the biological meaning and the potential active role of these
455 metabolites during fertilization; ii) the primary source of these compounds; iii) the mechanisms and

456 the energy expenditure allowing their accumulation in seminal plasma. If the role of purines
457 (particularly of adenosine) are strictly associated with the presence of purine receptors affecting
458 spermatogenesis, sperm maturation and fertilizing capacity (Bellezza and Minelli, 2017), it is not at
459 all clear the need for such high concentrations of these compounds in seminal plasma. Uridine,
460 guanosine, guanine and xanthine had significantly different values in infertile patients ($p < 0.001$
461 compared to fertile controls) strongly suggesting a potential imbalance in mitochondrial-related
462 energy metabolism and a direct involvement of purines and pyrimidines in the pathological
463 mechanisms of male infertility. In fact, studies in different pathological states linked the
464 fluctuations of these compounds to mitochondrial malfunctioning (Micheli et al., 2011; Sheeran and
465 Pepe, 2017).
466 The subsequent categorization of infertile patients into those without (normozoospermic) and with
467 (cumulative asthenoteratooligozoospermic + asthenozoospermic + teratozoospermic +
468 oligozoospermic) alterations on the spermiogram allowed to evidence apparently few differences in
469 the redox-energy evaluation of their seminal plasma. Only ascorbic acid, *all*-trans retinol, α-
470 tocopherol, cytidine, uridine and guanine were significantly different in the two subgroups of
471 patients (Table IV).
472 However, observing raw data of each patient it was tangible that infertile N and infertile ATO + A +
473 T + O might differ for the number of alterations of the 21 biomarkers distinguishing fertile from
474 infertile rather than for the actual concentrations of each of these analytes. The introduction of the
475 Biomarker Score, as a cumulative index of the biochemical anomalies in the quality of seminal
476 plasma, initially showed that fertile controls (Biomarker Score ranging between 0 and 4) and
477 infertile patients (Biomarker Score ranging between 5 and 21) were clustered into two clearly
478 distinguishable groups (Fig. 2). The further application of the Biomarker Score to infertile patients
479 categorized into infertile N and infertile ATO + A + T + O, permitted to evidence not only that
480 infertile ATO + A + T + O had higher mean values of the Biomarker Score ($p < 0.005$ compared to
481 infertile N), but also that the frequency of distribution of their Biomarker Score values appeared

different from that of infertile N (Fig. 3). By applying the Bayes theorem to calculate the posterior probability curves of fertile controls, infertile N and infertile ATO + A + T + O it was possible to observe that the use of the Biomarker Score permits to clearly distinguish 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 (Fig. 4). It is of particular relevance to underline that infertile N, i.e. the category of patients considered normal on the basis of morpho-functional analysis, had values of the Biomarker Score much higher from those of fertile controls indicating that biochemical/metabolic problems are very probably involved in their inability to reproduction.

In conclusion, results of this study evidenced that the present *Redox-Energy Test*, aimed to measure selected compounds representative of antioxidant defences, of oxidative/nitrosative stress damage, of purines, pyrimidines and energy metabolism, and having the biochemical meaning of biomarkers for make infertility, is a useful tool either to grade the severity of the metabolic alterations of human seminal plasma or in the decision making of subsequent personalized treatments of infertile males. With respect to the analyses currently in use for a gross evaluation of certain pathological phenomena (ROS damage and production, decrease of total antioxidant capacity) (Pahune et al., 2013; Agarwal et al., 2015; Bergamo et al., 2016; Roychoudhury et al., 2016; Riaz et al., 2016), that do permit neither to evaluate nitrosative stress, nor to measure qualitatively and quantitatively the different antioxidants, nor to determine eventual signs of energy imbalance (Pahune et al., 2013; Agarwal et al., 2015; Bergamo et al., 2016; Roychoudhury et al., 2016; Riaz et al., 2016), the analyses used in this study generate results that can help clinicians to start personalized treatments with specific adjuvant, nutraceuticals, diets, finalized to ameliorate the quality of seminal plasma. The *Redox Energy Test*, coupled with the Biomarker Score that cumulates the biochemical characteristics of seminal plasma into a single index, might be considered the correct analytical approach that should mandatorily be effected before any therapeutic strategy is undertaken (particularly, the indiscriminate administration of antioxidants with no indications on any specific deficiency).

Further studies to increase 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.

CONTRIBUTION OF AUTHORS TO THE PAPER

Giacomo Lazzarino contributed to sample preparation for HPLC analysis, carried out the HPLC determination of fat-soluble compounds and wrote in part the manuscript.

Ilaria Li Storti carried out spermiogram, contributed to sample preparation for HPLC analysis and revised the manuscript.

Luigi Muzii carried out spermiogram, performed collection of clinical data and revised the manuscript.

Angela M. Amorini carried out the HPLC determination of water-soluble compounds and revised the manuscript.

Salvatore Longo evaluated results of the HPLC analyses and revised the manuscript.

Enrico Di Stasio carried out the statistical tests and the introduction of the Biomarker Score and revised the manuscript.

Giuseppe Caruso evaluated results of the HPLC analyses, carried out basic statistic evaluation of the data and revised the manuscript.

Serafina D'Urso evaluated results of the HPLC analyses and revised the manuscript.

Giuseppe Lazzarino analyzed results and wrote the manuscript.

Barbara Tavazzi analyzed results and wrote the manuscript.

Pasquale Bilotta performed the clinical evaluation of the patients and revised the manuscript.

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CONFLICTS OF INTEREST

Authors have no conflicts of interest to declare.

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LEGEND TO FIGURES

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.

Figure 2. Box plot (A) reporting the values of the Biomarker Score obtained in the groups of controls and pooled infertile patients. Distribution frequency (B) of the Biomarker Score in the two groups. About the 94% of controls had Biomarker Score values from 0 to 3, with the remaining 6% scoring 4. Conversely, pooled infertile patients had Biomarker Scores ranging from 5 to 21, with more than the 89% of them ranging between 9 and 16.

*significantly different from controls, $p < 0.001$.

Figure 3. Box plot (A) reporting values of the Biomarker Score obtained in the groups of controls and infertile patients categorized into normozoospermic (N, n = 28) and asthenoteratooligozoospermic + astheno + terato + oligozoospermic (ATO + A + T + O, n = 68). Distribution frequency (B) of the Biomarker Score in controls and in the groups of patients categorized into those without (N) and with (ATO + A + T + O) anomalies on the spermiogram.

*significantly different from controls, $p < 0.001$.

**significantly different from controls, $p < 0.01$.

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