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Sperm ubiquitination positively correlates to normal morphology in human semen

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BACKGROUND: Global ubiquitination in human semen has been found to negatively correlate with standard semen parameters, indicating that ubiquitination can be considered a marker of poor semen quality. However, the inclusion of all semen components in the analysis may be misleading on the biological significance of ubiquitination of sperm cells. We have recently demonstrated the variable presence of bodies of different size, with the highest concentration in oligoasthenoteratozoospermia. The purpose of the present study was to evaluate the relationship between ubiquitination and standard semen parameters, after distinguishing between ubiquitinated sperm and bodies in each sample. METHODS: Ubiquitination was evaluated by flow cytometric sperm ubiquitin tag immunoassay (SUTI) in sperm samples from 45 subjects. Semen analysis was performed according to WHO (1999) guidelines. RESULTS: When only ubiquitinated sperm were considered, a positive correlation with number, motility and normal morphology was found. When correlation was evaluated considering the percentage of ubiquitinated bodies, a negative correlation was found with good semen quality. CONCLUSIONS: Results indicate that the negative correlations previously found between global semen ubiquitination and parameters of semen analysis are mainly driven by components other than sperm. The positive correlation between sperm ubiquitination and good quality parameters suggests a previously unrecognized role for sperm ubiquitination.

Key words: semen analyses/sperm morphology/ubiquitin

Introduction

Ubiquitin (Ub) is a small and highly conserved protein present in apparently all eukaryotic cells. Although ubiquitin exists as a free polypeptide, it has the property of binding covalently to other proteins, via an isopeptide bond between the C-terminal glycine of ubiquitin and the ε-amino group of a lysine in substrate proteins. Conjugation of ubiquitin (ubiquitination) to a protein can occur by the attachment of a single ubiquitin moiety (Hicke, 2001; Haglund *et al.*, 2003a) or of polymeric ubiquitin chains (Pickart, 2001). Polyubiquitination has the role of labelling proteins for degradation by the 26S proteasome or by lysosomes (Hochstrasser, 1996). Ubiquitin-mediated protein degradation is involved in the control of many events, including cell cycle progression (Hershko and Ciechanover, 1998), membrane receptor endocytosis (Haglund *et al.*, 2003b) and apoptosis (Friedman and Xue, 2004).

Recently, an important role for sperm protein ubiquitination has been postulated by the group of Sutovsky *et al.* (2001a). These authors proposed the existence of a ubiquitin mediated system, acting in the mammalian epididymis devoted to recognising and labelling defective sperm (Sutovsky *et al.*, 2001a). Indeed, they observed that the percentage of ubiquitinated sperm increases during the transit from the rete-testis to the corpus of epididymis, and decreases from the corpus to the cauda of epididymis (Sutovsky *et al.*, 2001a). In addition, they reported that epididymal epithelial cells secrete ubiquitin and phagocytose labelled sperm, both *in vivo* and in *in vitro* culture (Sutovsky *et al.*, 2001a). However, it is still to be resolved whether degradation of spermatozoa occurs through epithelial phagocytosis or by intraluminal degradation in the epididymal tubules. In this respect, it has been shown recently that proteasome is present in epididymal fluid in animals (Jones, 2004). Morphologically abnormal ubiquitinated sperm found in the ejaculate are considered to have escaped from epididymal phagocytosis and are characterized by main defects of head and/or axoneme (Sutovsky *et al.*, 2001a,b). Ubiquitinated sperm with normal morphology were also found (Sutovsky *et al.*, 2001b), although ubiquitin labelling appears to be weaker than in abnormal ones. Semen ubiquitination was found increased in patients affected by dysplasia of the fibrous sheath (Rawe *et al.*, 2002), while in bovine semen ubiquitination is partially associated with DNA fragmentation (Sutovsky *et al.*, 2002). Moreover, it has been demonstrated recently that semen ubiquitination is associated with poor quality sperm parameters (Sutovsky *et al.*, 2004), although in a previous paper, performed in a smaller number of subjects,

the same group reported the occurrence of a positive correlation between semen ubiquitination and sperm count and motility (Sutovsky *et al.*, 2001b). All the mentioned studies were carried out by using rough semen and without distinguishing between different components. Hence, the relationship found between ubiquitination in semen and standard semen parameters might be affected by the presence of ubiquitinated semen components other than sperm. Based on this speculation, the conflicting results mentioned above (Sutovsky *et al.*, 2001a, 2004) could be explained by an inter-sample variability in the relative amounts of the different semen components. Among these, we (Muratori *et al.*, 2004) have recently demonstrated the presence of round shaped bodies, [termed M540 bodies because of their labelling with merocyanine 540 (M540)], with the highest concentration in semen from oligoasthenoteratozoospermic (OAT) men. M540 bodies are virtually devoid of nuclear material, heterogeneous in size (including those similar to sperm heads) and density (Muratori *et al.*, 2004), showing similar characteristics to apoptotic bodies previously found in human semen from sub-fertile patients (Baccetti *et al.*, 1996; Gandini *et al.*, 2000). In the present study we show that M540 bodies are also ubiquitinated. In view of this result, we decided to study the relationship between ubiquitination and standard semen parameters, including only spermatozoa, in the analysis. In addition, since our previous data showed that occurrence of M540 bodies is associated with poor semen quality of semen samples (Muratori *et al.*, 2004) we verified whether the amount of ubiquitinated bodies could affect the association between poor quality semen and standard semen parameters (Sutovsky *et al.*, 2004).

Materials and methods

Chemicals

Human tubal fluid (HTF) medium was purchased from Celbio (Milan, Italy). Antibody KM 691 was from Kamyia Biomedical Company, Seattle, WA. FITC-conjugated goat anti-mouse IgM was from Zymed Laboratories, San Francisco, CA. Normal Goat serum (NGS) and the other chemicals were from Sigma Chemical Co (St Louis, MO).

Semen samples collection and preparation

Semen samples were collected [according to WHO criteria (WHO, 1999)] from 45 subjects undergoing routine semen analysis for couple infertility in the Andrology laboratory of the University of Florence after the approval of the Hospital Committee for Investigations in Humans and after informed patient consent. Sperm morphology and motility were assessed by optical microscopy, according to WHO criteria (WHO, 1999). Sperm morphology was scored by the determining percentage of normal and abnormal forms after Diff–Quik staining (WHO, 1999). Sperm motility was scored by the determining percentage of progressive motile, non progressive motile and immotile spermatozoa. Semen samples were consecutively collected from normozoospermic (N, $n = 14$)), teratozoospermic (T, $n = 11$), asthenoteratozoospermic (AT, $n = 9$) and oligoasthenoteratozoospermic (OAT, $n = 11$) subjects (WHO, 1999). Experiments were performed in rough semen samples after washing twice with HTF medium.

Flow cytometry

For detection of ubiquitin, samples were processed for Sperm Ubiquitin Tag Immunoassay (SUTI), as described by Sutovsky *et al.* (2001b) with minor modifications. Briefly, sperm suspensions were fixed in paraformaldehyde [200 µl, 4% in phosphate-buffered saline (PBS) pH 7.4] for 30 min at room temperature. After twice washing with 1% NGS–PBS, sperm were blocked for 25 min in 5% NGS in PBS and washed again. Then, samples were split into two aliquots: one was incubated for 40 min with the monoclonal antibody KM 691 raised against the recombinant human ubiquitin (dilution 1/100 in 1% NGS–PBS). The other aliquot was used to prepare negative controls (absence of primary antibody). After washing twice with 1% NGS–PBS, sperm were incubated in the dark for 40 min with FITC-conjugated goat anti-mouse IgM (dilution 1/80 in 1% NGS–PBS). Samples were washed twice, resuspended in 500 µl of PBS, stained with 10 µl of propidium iodide (PI, 30 µg/ml in PBS) and incubated in the dark for 15 min at room temperature. Samples were acquired by a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with a 15 mW argon-ion laser for excitation.

For each test sample, two sperm suspensions were prepared for instrumental setting and data analysis: (i) by omitting both primary antibody against ubiquitin and PI staining (negative sample); (ii) by omitting only the PI staining (sample for compensation, see below). Green fluorescence of FITC-conjugated goat anti-mouse IgM was revealed by an FL-1 (515–555 nm wavelength band) detector; red fluorescence of PI was detected by an FL-2 (563–607 nm wavelength band) detector. Quadrant setting was established in the FL-1/FL-2 dot plot corresponding to the negative sample, by including 99% of total events in the lower left quadrant. Fluorescence compensation was set by acquiring samples labelled by only SUTI. For each sample, 10 000 events were recorded within the characteristic flame shaped region in the Forward light scatter/Side light scatter (FSC/SSC) dot plot which excludes debris and large cells (R1 in Figure 1A; Muratori *et al.*, 2000, 2003, 2004). Percentages of ubiquitinated M540 bodies (Muratori *et al.*, 2004) were determined within such region, including both sperm and bodies (Muratori *et al.*, 2004). Percentage of ubiquitinated sperm were determined in the population formed solely by sperm, i.e. within the PI positive events of R1 region. To determine mean and median values of ubiquitin distribution, we gated DNA containing and not containing events within R1 region.

Immunofluorescence microscopy

Double stained samples for ubiquitin and nuclei (see above), were smeared on slides and examined using a fluorescence microscope (Leitz, Type 307-148002, Wetzlar, Germany) equipped with E4 and N2.1 filters (Leica, Milan, Italy) by an oil immersion 100× magnification objective. Images were captured by a Canon digital camera using Remote Capture software (provided by Canon, Japan) and edited by Adobe photoshop version 5.0 (Adobe Systems Inc., CA).

Statistic analysis

All variables were checked for normal distribution. Bivariate correlation was evaluated by calculating the Pearson's correlation coefficient (r). Multiple linear regression analysis was performed to establish which variable could explain a significant amount of variation in sperm ubiquitination. A stepwise procedure was used to select variables to be included into the model. A variable was entered into the model if the probability of F was smaller than 0.05 and removed if it was ≥ 0.1. All statistical analyses were carried out

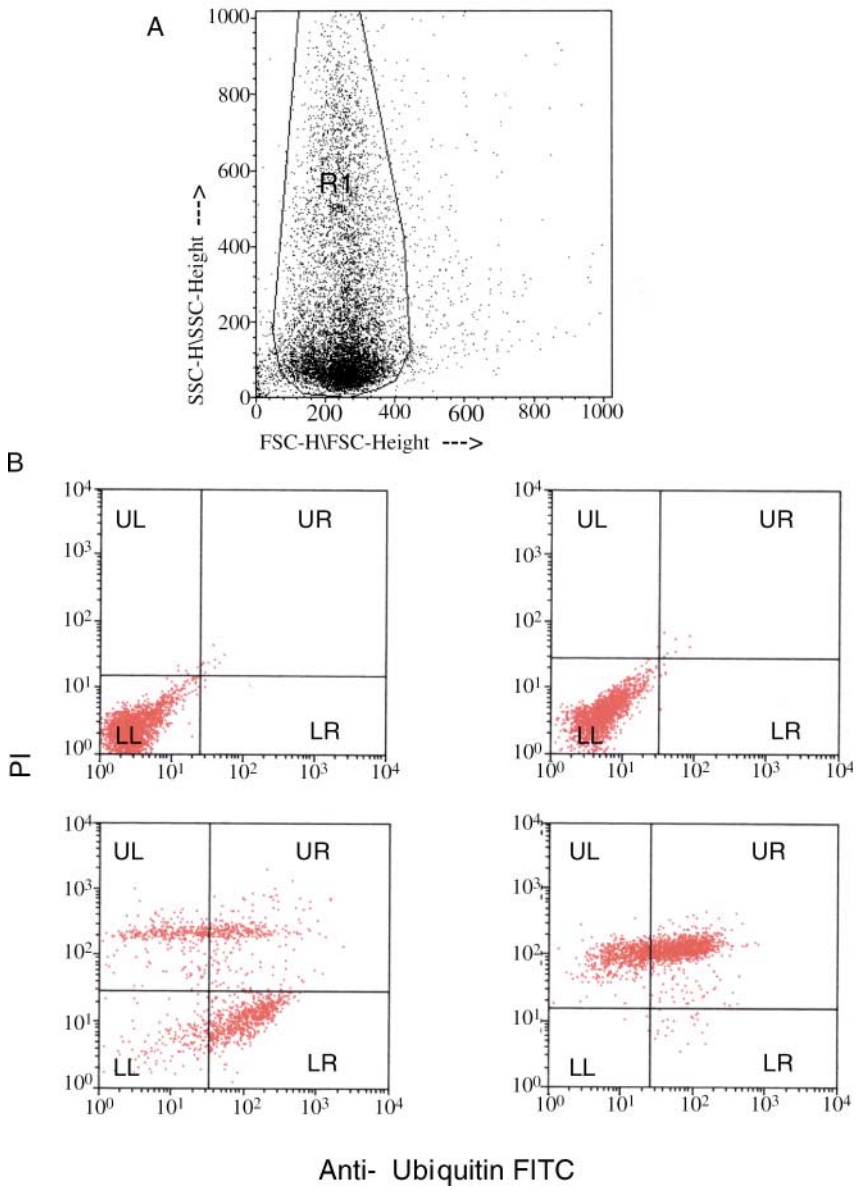

Figure 1. (**A**) FSC/SSC dot plot identifying the flame shaped region (R1) containing spermatozoa and M540 bodies. (**B**) Dot plots showing negative samples (negative controls for ubiquitin labeling obtained in the absence of KM-691 antibody and PI, upper panels) and corresponding test samples (obtained with double staining with SUTI and PI, lower panels) from an oligoasthenoteratozoospermic (left panels) and a normozoospermic (right panels) man. Lower panels: UR = Ub^+/PI^+, ubiquitinated sperm. UL = Ub^-/PI^+, not ubiquitinated sperm. LR = Ub^+/PI^-, ubiquitinated bodies. LL = Ub^-/PI^-, not ubiquitinated bodies. UR: Upper Right quadrant. UL: Upper Left quadrant. LR: Lower Right quadrant. LL: Lower Left quadrant. Ub: ubiquitin. PI: propidium iodide.

using the SPSS, version 11.5, software for Windows (SPSS Inc, Chicago, IL).

Results

We studied ubiquitination in human semen by SUTI coupled to flow cytometry. FACScan analysis of semen ubiquitination was performed within the flame shaped region which excludes debris and large cells (Figure 1A; Muratori *et al.*, 2003, 2004). As outlined above, we have shown that such region includes round bodies (M540 bodies) devoid of chromatin material (Muratori *et al.*, 2004). Thus, to discriminate between such bodies and sperm, we labelled samples also with the nuclear stain PI. Figure 1B shows typical dot plots obtained by double staining with SUTI and PI in an OAT (left panels) and an N (right panels) man, characterized, respectively, by high and low levels of M540 bodies (Muratori *et al.*, 2004). Upper panels represent the corresponding negative controls for ubiquitin labelling (omitting primary anti-ubiquitin antibody). As shown in the lower panels (test samples), a large percentage of M540 bodies were ubiquitinated (LR quadrants) together with a consistent percentage of spermatozoa (UR quadrants) in both samples. To confirm that M540 bodies are ubiquitinated, double stained samples for SUTI and PI were observed by fluorescence microscopy (Figures 2 and 3). Figure 2 was obtained from three OAT (characterized by a high percentage

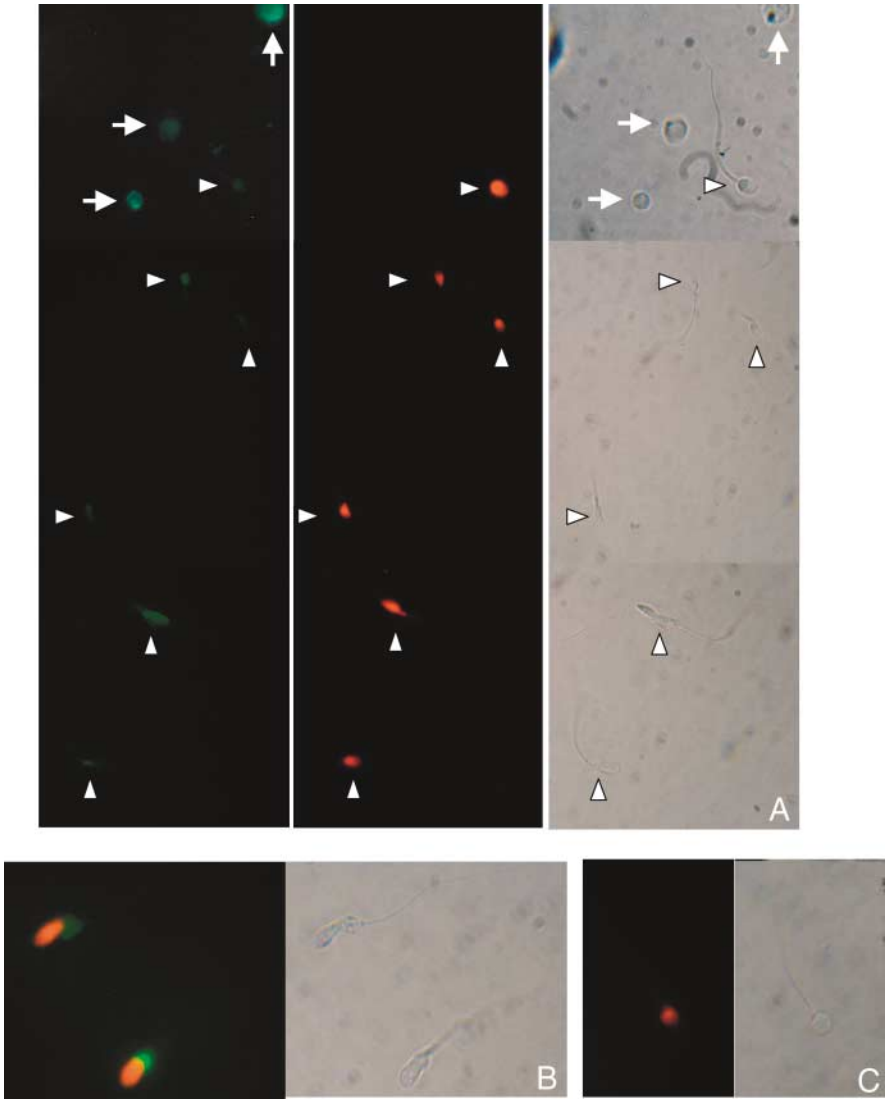

Figure 2. Micrographs of semen sample from three oligoasthenoteratozoospermic men by fluorescence microscopy after double staining with SUTI and PI. (**A**) Green fluorescence (ubiquitin labelling, left panels), red fluorescence (nuclei labelling, middle panels) and bright images (right panels) from the same field are shown. Arrowheads indicate sperm, arrows indicate bodies. (**B** and **C**) The image (left panels) has been obtained by overlapping the two fluorescent signals described for (**A**). Bright images corresponding to the same fields are shown in the right panels.

of M540 bodies; Muratori *et al.*, 2004), whereas Figure 3 was from two N subjects. Figure 2A shows green fluorescence from ubiquitin labelling (left) and red fluorescence from PI staining of sperm nuclei (middle) from the same dark fields, while images of panels B and C have been obtained by overlapping the two fluorescent signals. The corresponding bright fields are shown on the right. As can be observed, both sperm (PI labelled, arrowheads) and bodies (lacking PI labelling, arrows) were ubiquitinated. Labelling for ubiquitin was localized mainly in the mid piece region and, occasionally, in the head of morphologically abnormal spermatozoa (Figure 2A). In addition, cytoplasmic droplets were always intensively labelled when present (Figure 2A and B). Non ubiquitinated abnormal sperm were occasionally found (Figure 2C, round head sperm). In Figure 3A and B, green fluorescence of ubiquitin labelling and the corresponding bright fields are shown, while in panels C–E, fluorescence images have been obtained by overlapping the PI (red) and ubiquitin (green) signals. Again, labelling was mainly apparent in the mid-piece region of morphologically abnormal sperm (indicated by arrowheads in panels A and C). Apparently morphologically normal spermatozoa were always labelled in the mid-piece, even if labelling in the principal and in the end pieces of the tail (panels B–E, non indicated sperm) was also frequently present. Overall, these results demonstrate that M540 bodies (Muratori *et al.*, 2004) are ubiquitinated and confirm that labelling for ubiquitin is present both in defective and in normal spermatozoa (Sutowsky *et al.*, 2000, 2001b).

As mentioned above, it has been demonstrated recently that ubiquitination in whole human semen is related to poor quality of sperm parameters (Sutovsky *et al.*, 2004). In view

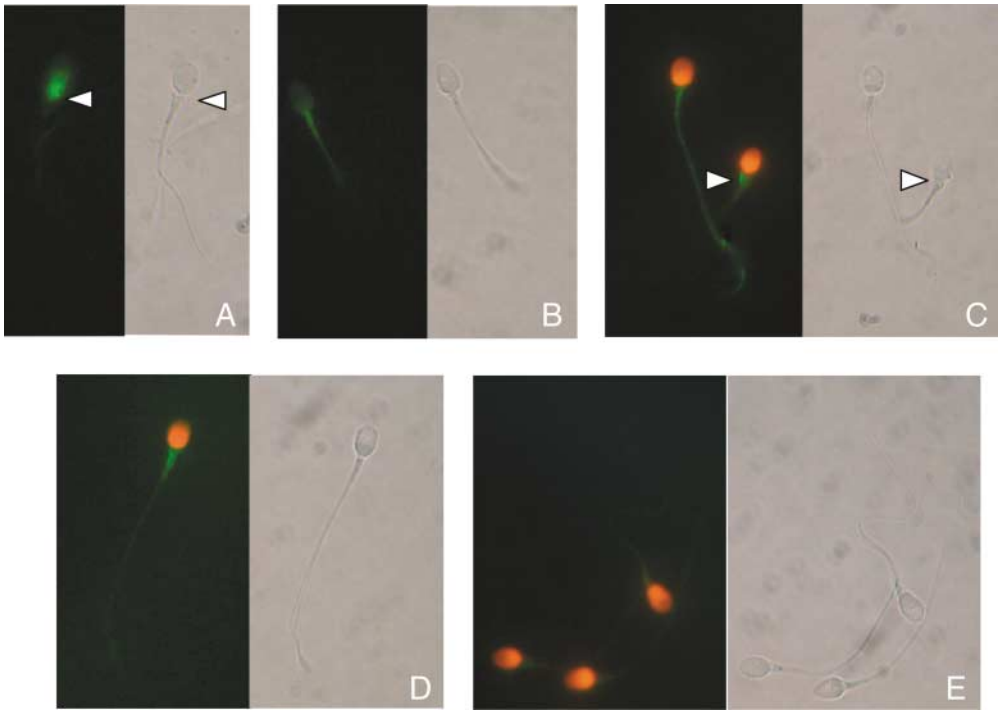

Figure 3. Micrographs of semen samples from two normozoospermic men by fluorescence microscopy after double staining with SUTI and PI. (**A** and **B**) Green fluorescence (ubiquitin labelling, left panels) and bright images (right panels) from the same field are shown. (**C**–**E**) The image has been obtained by overlapping the fluorescent signals of PI (nuclei labelling, red) and SUTI (ubiquitin labelling, green). Bright images corresponding to the same fields are shown in the right panels. Arrowheads indicate morphologically abnormal sperm.

Table I. Semen parameters, age and abstinence (mean ± SD) of subjects included in the present study

Subject parameters	N ($n = 14$)	AT ($n = 9$)	T ($n = 11$)	OAT ($n = 11$)
Sperm count ($\times 10^6$)	260.2 ± 145.3	226.6 ± 200.9	234.5 ± 97.3	33.2 ± 23.8
Sperm concentration (sperm/ml)	119.1 ± 101.5	64.8 ± 32.9	88.4 ± 52.2	9.6 ± 5.1
Total motility (%)	74.2 ± 10.6	57.9 ± 13.7	74.4 ± 6.6	41.2 ± 19.7
Progressive motility (%)	59.9 ± 9.9	32.2 ± 13.6	58.9 ± 7.1	17.9 ± 14.5
Normal morphology (%)	35.6 ± 6.5	16.2 ± 6.1	21.0 ± 7.5	7.5 ± 4.7
Volume of the ejaculate	2.9 ± 1.0	3.5 ± 2.2	2.9 ± 1.0	3.8 ± 2.0
pH	7.4 ± 0.1	7.4 ± 0.2	7.2 ± 0.2	7.5 ± 0.2
Age (years)	34.2 ± 6.6	31.8 ± 8.4	35.6 ± 4.9	35.0 ± 12.1
Abstinence (days)	4.1 ± 1.3	4.8 ± 1.1	4 ± 0.9	3.0 ± 1.1

N = normozoospermic, T = teratozoospermic, AT = asthenoteratozoospermic, OAT = oligoasthenoteratozoospermic subjects.

of our results demonstrating that M540 bodies are ubiquitinated (Figures 1 and 2) we calculated such a relationship in 45 subjects (whose standard semen parameters are shown in Table I) by separately considering the percentages of ubiquitinated sperm and bodies.

First, we determined the percentage of ubiquinated sperm by excluding bodies [i.e. the percentage of events in the UR quadrants on total sperm population (UL plus UR quadrants, Figure 1B, test samples)]. To our surprise, when these values were correlated to standard semen parameters, positive correlations between sperm ubiquitination and sperm count (Figure 4A), concentration (Figure 4B), motility (total and progressive, Figure 4C and D, respectively) and normal morphology (Figure 4E) were found. Correlation coefficients and significance for correlation analysis are shown in Table II. To identify which independent variable (among semen parameters) explained a significant amount of the variation in percentage sperm ubiquitination, we used a stepwise multiple linear regression model. This analysis demonstrated that only the percentage of normal morphology satisfied the criteria to be included in the model. In other words, morphology is the semen parameter that mainly (positively) affects sperm ubiquitination. We analysed the relationship between standard semen parameters and sperm ubiquitination, by also considering the median value of ubiquitin distribution (within the DNA containing events of R1 region, UL + UR quadrants, Figure 1B). A positive correlation with sperm concentration ($r = 0.3$, $P < 0.05$, $n = 45$) and sperm normal morphology ($r = 0.3$, $P < 0.05$, $n = 45$) was found. No significant correlation was found with the other tested semen parameters. In Table III, average mean and median of sperm ubiquitin distribution for the 45 men included in the study are reported.

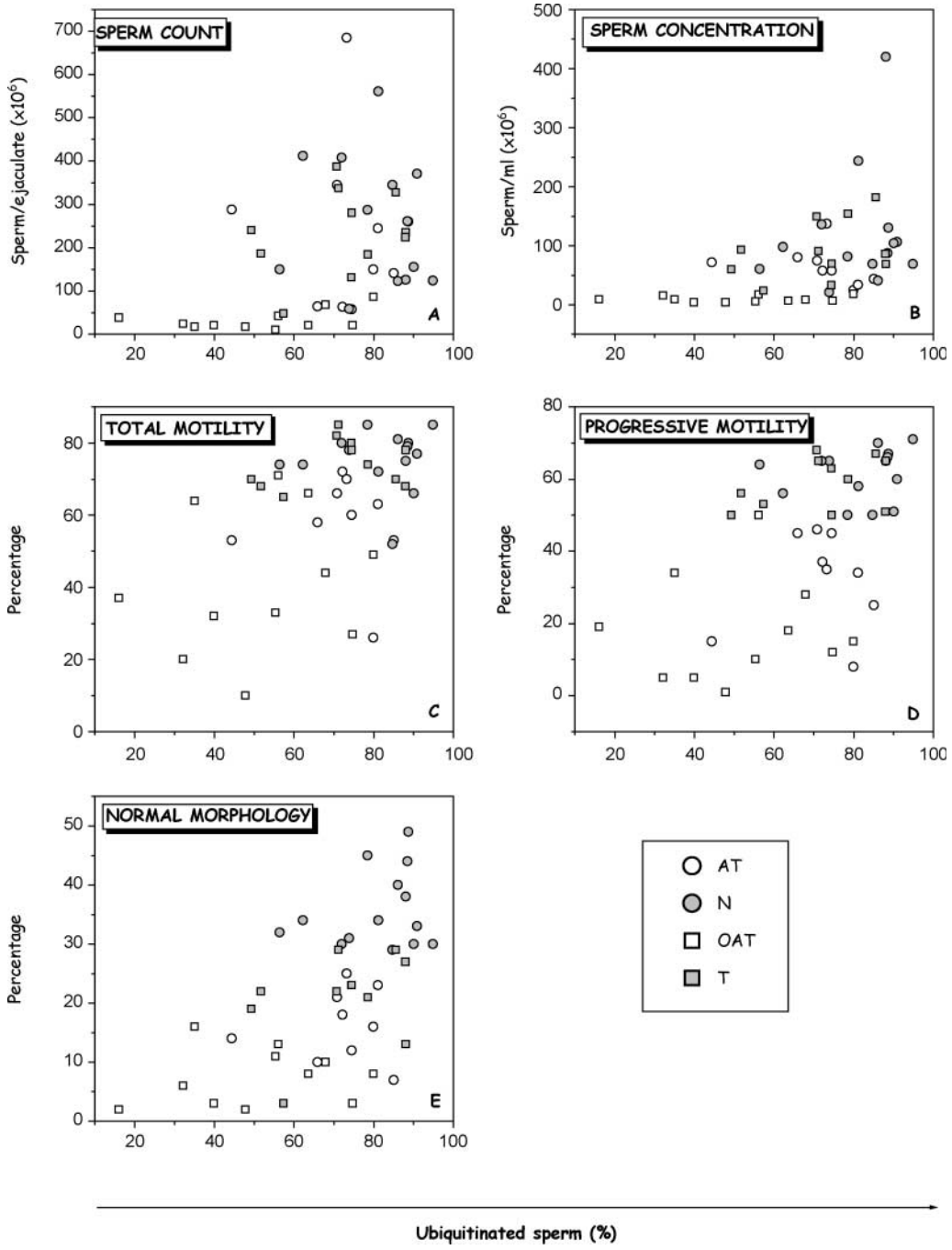

Figure 4. Scatter plots between percentage of ubiquitinated sperm from 45 semen samples, and sperm count (**A**), concentration (**B**), percentage total (**C**) and progressive (**D**) motility and percentage normal morphology (**E**). Pearson's correlation coefficients are reported in Table II. AT, asthenoteratozoospermic; N, normozoospermic; OAT, oligoasthenoteratozoospermic; T, teratozoospermic subject.

Table II. Correlation analysis of the relationship between the percentages of ubiquitinated sperm (mean ± SD = 69.8 ± 17.9; range: 16.0–94.9%) and standard semen parameters and correlation analysis of the relationship between the percentages of ubiquitinated bodies (mean ± SD = 12.4 ± 9.1; range: 2.3–44.2%) and standard semen parameters

Variable	Mean ± SD	*n*	Ubiquitinated sperm		Ubiquitinated bodies	
			r	*P* <	*r*	*P* <
Sperm count (sperm/ejaculate)	191.7 ± 156.0	4	0.36	0.05	−0.50	0.05
Sperm concentration (sperm/ml)	74.8 ± 74.9	4	0.42	0.01	−0.52	0.01
Total motility (%)	62.9 ± 19.0	4	0.48	0.005	−0.37	0.005
Progressive motility (%)	43.9 ± 21.4	4	0.52	0.001	−0.45	0.001
Normal morphology (%)	21.3 ± 12.5	4	0.58	0.001	−0.47	0.001

r, Pearson's correlation coefficient.

Table III. Average sperm (UL+UR quadrants) and bodies (LL+LR quadrants) ubiquitin mean and median values for the 45 subjects included in the study

	Mean ± SD	Median ± SD
Sperm	77.3 ± 32.4	50.8 ± 23.5
Bodies	111.5 ± 65.4	64.0 ± 9

As stated above, a large percentage of bodies were ubiquitinated (mean ± SD = 12.4 ± 9.1%, $n = 45$, events in LR quadrants in populations formed by sperm + bodies; Figure 1B) even if non ubiquitinated bodies are also present (mean ± SD = 10.3 ± 11.1%, $n = 45$, events in LL quadrants in populations formed by sperm + bodies; Figure 1B). When the percentage of ubiquitinated bodies was correlated to standard semen parameters, a negative correlation with sperm count (Figure 5A), sperm concentration (Figure 5B), motility (total and progressive, Figure 5C and D, respectively) and normal morphology (Figure 5E) was found. Correlation coefficients and significance for correlation analysis are shown in Table II. We analysed the relationship between standard semen parameters and bodies ubiquitination, also by

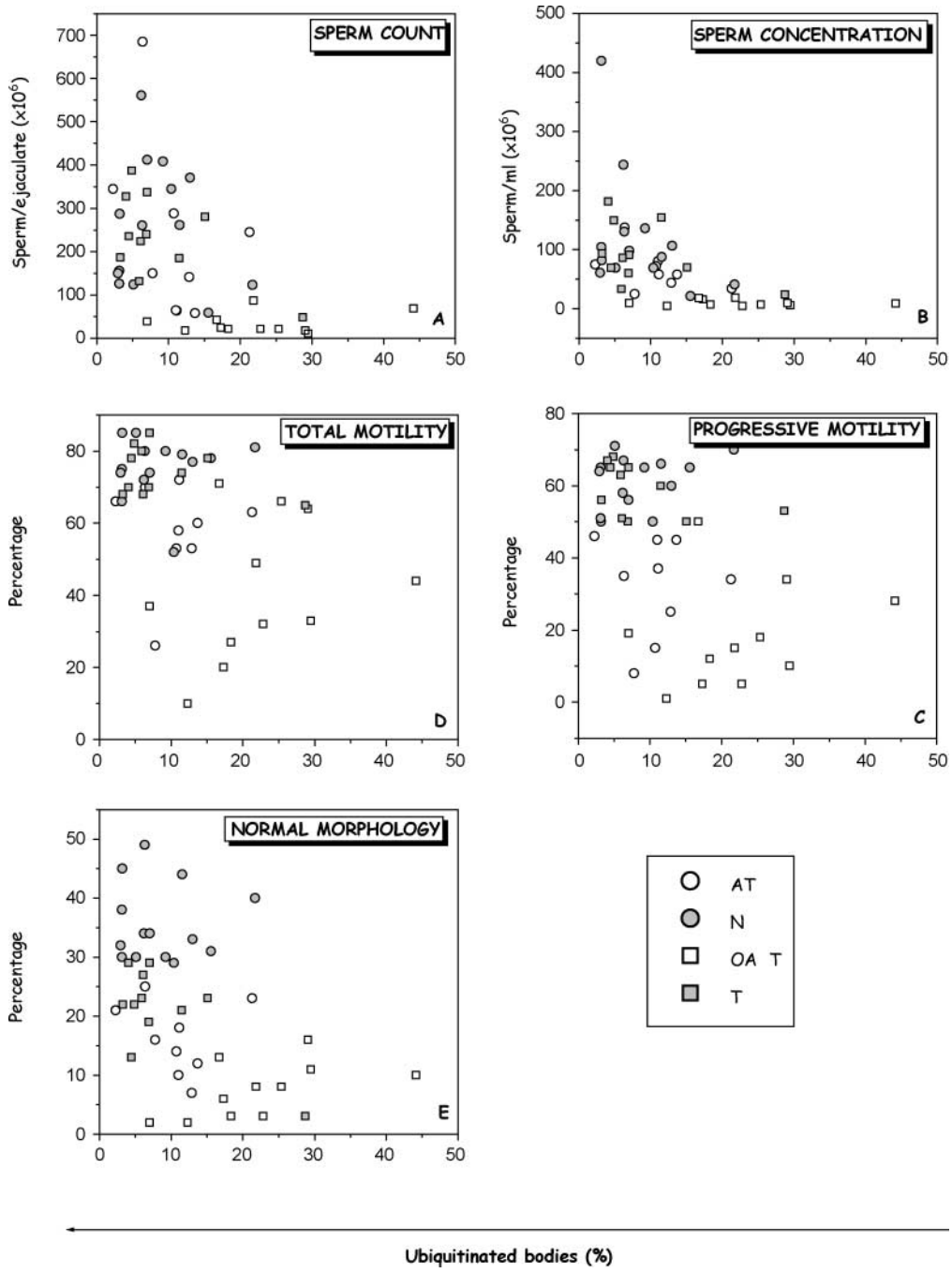

Figure 5. Scatter plots between percentage of ubiquitinated bodies from 45 semen samples and sperm count (**A**), concentration (**B**), percentage total (**C**) and progressive (**D**) motility and percentage normal morphology (**E**). Pearson's correlation coefficients are reported in Table II.

considering the median values of ubiquitin distribution (within the events not containing DNA in R1 region, LL + LR quadrants; Figure 1B). In this case, no significant correlation was found with any of the standard semen parameters. Average values of mean and median of ubiquitin distribution in bodies population are reported in Table III.

Discussion

The main message conveyed by the present study is that sperm ubiquitination is related to good quality of semen parameters, implying that ubiquitination of sperm proteins may have previously unrecognized additional biological meanings besides marking defective spermatozoa for their elimination during epididymal transit (Sutovsky *et al.*, 2001a). Indeed, in the present study we show that both morphologically abnormal and apparently morphologically normal (Figures 2 and 3) spermatozoa are ubiquitinated, confirming previous results (Sutovsky *et al.*., 2001a,b). Moreover, when the percentage of ubiquitinated sperm (excluding other semen components such as debris, M540 bodies, immature germ cells and leukocytes) in the 45 patients included in our study was related to standard parameters of semen analysis, a positive correlation was found with good quality of sperm parameters. Among standard semen parameters, the variable mainly affecting sperm ubiquitin was normal morphology, as suggested by the stepwise multiple linear regression analysis. When sperm ubiquitin was measured as the median value of ubiquitin distribution, the positive correlation was maintained with normal morphology and sperm concentration. This result suggests that the negative correlation previously found (Sutovsky *et al.*, 2004) between global semen ubiquitination and good quality semen (especially normal morphology) is mainly driven by components other than sperm included in the calculation. On the other hand, the authors report that an increase in the proportion of 'small cells/cellular debris (mostly of spermatogenic origin)' in the sample with high ubiquitin levels and reduced sperm count is present (Sutovsky *et al.*, 2004). In addition, the same authors demonstrated previously that elements other than spermatozoa are ubiquitinated, including unresorbed residual bodies and fragmented or sperm associated residual cytoplasm (Sutovsky *et al.*, 2001b). Here, we confirm that cytoplasmic residues are ubiquitinated and demonstrate that M540 bodies (Muratori *et al.*, 2004) are also ubiquitinated. In the present study, we also attempted to identify which semen components, other than sperm, might be responsible for the observed association between poor quality semen and overall ubiquitination. In this view, we verified whether M540 bodies might be responsible for such a relationship. Indeed, M540 bodies were ubiquitinated (present study) and particularly frequent in OAT subjects (Muratori *et al.*, 2004). We measured the amount of ubiquitin associated to bodies both as a percentage of ubiquitinated bodies (in R1 region) and as the median value of ubiquitin distribution in bodies population. Our results indicate that when percentages were considered, a negative correlation with standard parameters of seminal analysis was found. On the contrary, median values of ubiquitin distribution did not show any significant correlation with the same parameters. These findings may not be necessarily in contrast, since the two measures (percentage of ubiquitinated bodies and median ubiquitin values) refer to different populations (respectively population formed by sperm+bodies and population formed only of bodies). In any case, the finding that the median values of ubiquitin distribution within bodies population do not correlate with poor standard parameters does not allow us to definitively conclude that M540 bodies are the semen components which drive the association between overall ubiquitination and poor quality semen (Sutovsky *et al.*, 2004). Further studies will be necessary to address which semen component(s) (other than sperm) drive such associations.

The nature of M540 bodies has not been fully clarified. In our previous paper (Muratori *et al.*, 2004) we suggested that these elements resemble apoptotic bodies previously identified in semen from subfertile patients (Baccetti *et al.*, 1996; Gandini *et al.*, 2000). The finding that both these bodies and sperm-associated cytoplasmic residues are ubiquitinated (present study; Sutovsky *et al.*, 2001b), suggests that they might be cytoplasmic remnants detached from sperm. The two hypotheses are not completely in contrast, since it has been previously suggested that the mechanism responsible for the formation of apoptotic bodies is similar to that for residual bodies (Blanco-Rodriguez and Martinez-Garcia, 1999).

The finding of a positive correlation between percentages of ubiquitinated sperm (calculated after gating excluding debris, M540 bodies and other semen components) and semen parameters, especially normal morphology, suggests that ubiquitination may have a positive role in sperm function. The finding that morphologically normal sperm are also apparently ubiquitinated (Sutovsky *et al.*, 2000, 2001b; present paper), on the other hand, is consistent with the interpretation that the process of ubiquitination is not only devoted to mark defective sperm but may also have other roles. In this perspective, it has been reported that ubiquitination in sperm mitochondria may be responsible for the degradation of these organelles by the oocyte after fertilization (Sutovsky *et al.*, 1999,2000). It is possible that morphologically normal and abnormal sperm are characterized by a different pattern of ubiquitin distribution, as also suggested by our data (Figures 2 and 3). Indeed, when observed by fluorescence microscopy, a difference in the localization of ubiquitin labelling is apparent between normal and abnormal sperm: in the latter the tail is not or only occasionally ubiquitinated, whereas structures such as cytoplasmic residues and the mid piece are more intensely labelled. On the contrary, in apparently normal ubiquitinated spermatozoa, the principal piece of the tail was frequently labelled. Similarly, tail ubiquitination has been detected in apparently normal spermatozoa by Sutovsky *et al.* (2001b). It is possible that ubiquitination in normal and abnormal spermatozoa occurs in different proteins, with different biological significance. Indeed, anti-ubiquitin antibodies may virtually detect all ubiquitinated proteins (Pickart, 1998). In addition, it has been shown that, when tails and head from bovine sperm are separated by fractionation, different ubiquitinated protein bands are found, suggesting the presence of different substrates in the two structures

(Sutovsky *et al.*, 2000). Whether ubiquitination in different sperm structures has a different biological role remains to be determined.

In conclusion, we propose here another role for sperm ubiquitination besides marking of defective spermatozoa (for their elimination as originally proposed by Sutovsky *et al.*, 2001a). Our data indeed indicate that ubiquitination is also a normal sperm property, which positively correlates with normal morphology.

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