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# Decrease of air pollution during lockdown in Tuscany (Italy): An effect on sperm DNA fragmentation?

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## Abstract

In March 2020, the Italian government imposed a national lockdown which was almost completely removed in June 2020. Due to the abrupt stop of human activities, emissions of air pollutants decreased. Air pollution is an environmental risk factor for noncommunicable disease and mortality. Emerging evidence also suggests a role in male infertility. In this study, we compared sperm DNA fragmentation (sDF) levels and conventional semen parameters between subjects undergoing sDF determination and routine semen analysis in a single Italian centre, during about 6 months before ( $N = 119$ ) and after lockdown ( $N = 105$ ). After lockdown, we found an improvement of sperm progressive motility (48.00[38.50–58.00]% vs. 42.00 [33.00–53.00]%) and sDF levels (as total: 24.79[18.33–33.97]% vs. 35.02 [25.04–45.73]%,  $p < .001$ ; brighter: 14.02[10.69–17.93]% vs 18.54[13.58–25.82]%,  $p < .001$  and dimmer sDF: 9.24[5.64–15.78]% vs. 12.24[8.08–19.10]%,  $p < .01$ ), mirrored by a decrease of leukocyte semen concentration ( $p < .01$ ). The improvement of sperm motility and DNA quality was maintained after adjusting for leukocyte concentration and several conditions known to affect sperm motility and/or sDF levels. With a significant decrease in air pollution observed in Tuscany during and after lockdown, associated improvement in sperm motility and DNA quality in patients referred to the infertility clinic is suggestive of the potential role of air pollution in male infertility.

## KEYWORDS

air pollution, conventional semen parameters, lockdown, male infertility, sperm DNA fragmentation, TUNEL/PI assay

## 1 | INTRODUCTION

In early 2020, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) started spreading across the world. In the attempt to contain the spread of the disease, many countries imposed national

lockdowns, leading to an abrupt and unforeseen stop of most economic and social activities. In Italy, the government imposed a national lockdown on March 9, 2020, drastically reducing the movement of roughly 60 million people, except for work or health reasons, and closing schools and nonessential socioeconomic activities. Such sharp

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restriction ended in early May 2020, when human activities gradually restarted and came back to near normality in June 2020.

As a consequence of the stay-at-home order, emissions of air pollutants suddenly decreased in many countries (Uday et al., 2022; Wu et al., 2022) offering a real-world and unique test to evaluate the effect of anthropogenic activities on environmental pollution levels.

Air pollution poses a major threat to health, being a leading environmental risk factor for noncommunicable disease (Murray et al., 2020) and for mortality. In particular, it has been calculated that outdoor air pollution caused 2.9, 4.2, 4.5 million premature deaths in 2000, 2015, and 2019, respectively, indicating a substantial increase over the past 20 years on a global scale (Fuller et al., 2022). Premature deaths attributable to air pollution are mainly caused by heart disease, stroke, lung diseases, and lung cancer (Fiordelisi et al., 2017; World Health Organization (WHO), 2016).

Air pollution may also have a role in male infertility and in the sharp world-wide decline of sperm count observed in subjects with an unknown fertility state (Levine et al., 2017, 2022). Indeed, albeit inconclusive, several studies using semen quality as a proxy to evaluate male potential fertility, found that exposure to air pollutants was associated with significant decreases in sperm concentration (Sokol et al., 2006; Wu et al., 2017), sperm count (Santi et al., 2016; Wu et al., 2017), sperm morphology (Hammoud et al., 2010; Hansen et al., 2010; Lao et al., 2018; Zhou et al., 2014), and total/progressive sperm motility (Hammoud et al., 2010; Selevan et al., 2000; Song et al., 2013). Beside conventional semen parameters, sperm DNA quality, usually expressed as sperm DNA fragmentation (sDF), has been emerging as an important marker for male fertility. SDF is a sperm anomaly better predicting natural conception than conventional semen parameters (Giwerzman et al., 2010; Muratori, Marchiani, et al., 2015) and also impacting reproductive outcomes in couples treated by IVF and ICSI (Cissen et al., 2016; McQueen et al., 2019; Tan et al., 2019). Although not confirmed by all studies (Hansen et al., 2010; Radwan et al., 2016), several authors reported that air pollutants are able to increase sDF. Bosco et al. found that when standards were exceeded by annual average values (up to  $39 \mu\text{g}/\text{m}^3$  for  $\text{PM}_{2.5}$ ) or in some days in a year, sDF increased in respect to values found in control areas (Bosco et al., 2018). An impact on sDF was reported also by a longitudinal study with repeated measures over 2 years, showing a direct association between sDF and levels of  $\text{SO}_2$ ,  $\text{NO}_x$ , and  $\text{PM}_{10}$  (Rubes et al., 2005). Further, an increase of sDF was recently reported when the major traffic-associated pollutants reached the highest concentrations ( $25 \mu\text{g}/\text{m}^3$  for  $\text{PM}_{2.5}$ ) in a winter season (Rubes et al., 2021). Finally, workers exposed to traffic pollution exhibited higher sperm DNA damage than unexposed control men (Calogero et al., 2011).

The present study aims to evaluate whether, after lockdown, changes occurred in sDF (primary endpoint) and in conventional semen parameters (secondary endpoint), when considering male partners of infertile couples of a Central Italy region, Tuscany. To this aim, we compared levels of sDF and semen parameters between the two groups of subjects referring to a single center before and after the lockdown period. Results are discussed in light of the

decrease in air pollution which occurred during lockdown, as assessed in Tuscany area.

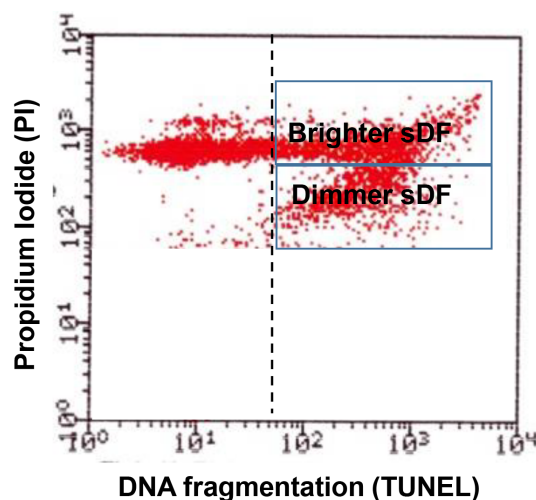
## 2 | MATERIALS AND METHODS

### 2.1 | Subjects

Patients undergoing semen analysis and sDF determination at the Andrology Laboratory of Careggi Hospital in about 6 months before (September 4–March 4, 2020) and after (May 13–November 25, 2020) lockdown were included in the study, after signing written informed consent. Patients were male partners of infertile couples. Female factors of infertility in these couples were unknown. In the period March 4–May 13, 2020, the service of semen analysis and sDF determination was discontinued according to the hospital guidelines for lockdown period. We excluded patients: (i) affected by oncological diseases, (ii) who had recently (within 2 years from day of sDF determination) undergone chemo- or radiotherapies, (iii) with azoospermia, and (iv) with insufficient sperm number for sDF detection. In total, from the initial 270 patients, we recruited 224 subjects for the study (119 in the pre- and 105 in the post-lockdown period). The study has been approved by the ethical committee of AOU Careggi (protocol no. 21279\_BIO).

### 2.2 | Semen analysis

Semen samples collection and semen analysis were conducted according to WHO criteria (WHO, 2010). Semen volume was evaluated by weighing the sample; sperm concentration was determined, in formalin diluted samples, using a Neubauer improved cell counting chamber and then was multiplied by semen volume to obtain sperm number/ejaculate; sperm motility was graded in progressive, nonprogressive, and immotile spermatozoa by scoring at least 200 cells; sperm morphology was evaluated after Diff-Quick staining in at least 200 spermatozoa. Semen pH was determined by spreading a drop of sample on a pH paper and comparing the obtained color with the calibration strip. To detect leukocytes, we deposited  $10 \mu\text{L}$  of semen on pre-stained slides Testsimplets® (AB Analitica, Padua, Italy), which were then evaluated at microscope with a 100X objective. The Andrology Laboratory of Careggi Hospital participates in external quality control programs: United Kingdom National External Quality Assessment Service (NEQAS) and External Quality Assessment of Tuscany. To patients undergoing sDF determination, a structured questionnaire was routinely administrated in order to collect information about: daily sedentary time, professional exposure to toxicants (pesticides, organic solvents, petroleum derivatives, Pb, Cd, radiation) or high temperature, smoking habits, daily alcohol consumption, supplement consumption, weekly physical exercise, history of cryptorchidism, mumps, varicocele, occurrence of inguinal hernia/testicular torsion, recent (within 6 months) urogenital infections, medications, current tumor, current disease, recent (within 2 years from day of sDF determination) chemo-radiotherapy.



**FIGURE 1** TUNEL/PI dot plot of a native semen sample. Note as nuclear staining identifies two different sperm populations (brighter and dimmer), the dashed line indicates the vertical marker established in the negative control and beyond which spermatozoa were considered DNA fragmented.

## 2.3 | SDF determination

SDF was detected in native semen samples with the TUNEL/PI technique. After two washes with Human Tubal Fluid medium and fixation with 200  $\mu$ L of 4% paraformaldehyde (30 min at RT), fixed samples were immediately processed with the In Situ Cell Death Detection Kit (Sigma Aldrich, Milan, Italy) as described elsewhere (Muratori et al., 2010). Briefly, after removing paraformaldehyde and washing twice with 200  $\mu$ L PBS/1% Bovin Serum Albumin, samples were permeabilized with the use of 100  $\mu$ L of 0.1% Triton X-100/sodium citrate for 4 min in ice. After washing twice, samples were treated with 50  $\mu$ L of labelling solution (supplied by the kit) containing the terminal deoxynucleotidyl transferase (TdT) enzyme (1 h at 37°C in the dark). After twice washing, samples were resuspended in 500  $\mu$ L PBS, stained with 10  $\mu$ L of propidium iodide (PI, 30 mg/mL in PBS) and incubated in the dark for 10 min at room temperature. Samples were acquired with a FACScan flow cytometer (Becton Dickinson) equipped with a 15-mW argon-ion laser for excitation. For each patient, three sperm suspensions were prepared, by omitting both PI staining and TdT (nonspecific fluorescence), omitting only TdT (negative control), and omitting only PI staining (for fluorescence compensation). TUNEL/PI allows discrimination of two sperm populations, namely brighter and dimmer sperm populations (Muratori et al., 2008; Figure 1).

Green fluorescence of nucleotides was revealed with an FL-1 (515–555 nm wavelength band) whereas red fluorescence of PI was detected with an FL-2 (563–607 nm wavelength band) detector. For each sample, we recorded 10,000 events within a FSC/SSC flame-shaped region (FR) characteristic of spermatozoa (Muratori et al., 2010). SDF was measured by gating the nucleated events (PI positive events) within the FR region (Muratori et al., 2010). This strategy allows analysis of only spermatozoa (Muratori et al., 2008), excluding

debris, large cells, and semen apoptotic bodies (Marchiani et al., 2007). For data analysis, we discriminated dimmer and brighter sperm populations with a horizontal marker in the PI axis of the TUNEL/PI dot plots; then a vertical marker was established in the TUNEL axis of the dot plot of negative control (TdT omitted), excluding 1% of total events. Such marker was copied in the dot plots of corresponding test samples, and all events beyond it were considered DNA fragmented spermatozoa. Dimmer sDF is the percentage of all dimmer spermatozoa [(dimmer DNA sperm/total sperm population)  $\times$  100], as they are all DNA fragmented; brighter sDF is calculated as: (brighter DNA fragmented sperm/total sperm population)  $\times$  100 (Muratori et al., 2008). Total sDF is dimmer sDF + brighter sDF.

## 2.4 | Data on air pollution

Data on air pollution were collected from online database of the Tuscany environmental protection agency (Agenzia Regionale per la Protezione Ambientale della Toscana, ARPAT ([https://www.arpat.toscana.it/temi-ambientali/aria/qualita-aria/grafici\\_bollettino](https://www.arpat.toscana.it/temi-ambientali/aria/qualita-aria/grafici_bollettino))) which provides a daily collection of PM<sub>10</sub>, PM<sub>2.5</sub>, NO<sub>2</sub>, O<sub>3</sub>, CO, SO<sub>2</sub>, H<sub>2</sub>S, and C<sub>6</sub>H<sub>6</sub> data by each station of the monitored area. Each station gives the 24-h average level. To estimate the daily concentration of each pollutant, we calculated the average across the stations of Tuscany, whereas to estimate overall exposure, we calculated the average over the indicated period. To verify whether a decrease of air pollution, did occur in Tuscany region due to lockdown, we compared the levels of the above air pollutants in the two periods: March 9–June 11, 2019 and March 9–June 11, 2020. To verify whether there was a different air pollution exposure of the pre- versus post-lockdown group, we compared levels of PM<sub>10</sub>, PM<sub>2.5</sub>, NO<sub>2</sub>, O<sub>3</sub>, CO, SO<sub>2</sub>, H<sub>2</sub>S, and C<sub>6</sub>H<sub>6</sub> in the two periods: June 1, 2019–February 29, 2020 and March 1, 2020–November 30, 2020 (each period starting 90 days before beginning sample collection in each group, considering the length of spermatogenesis).

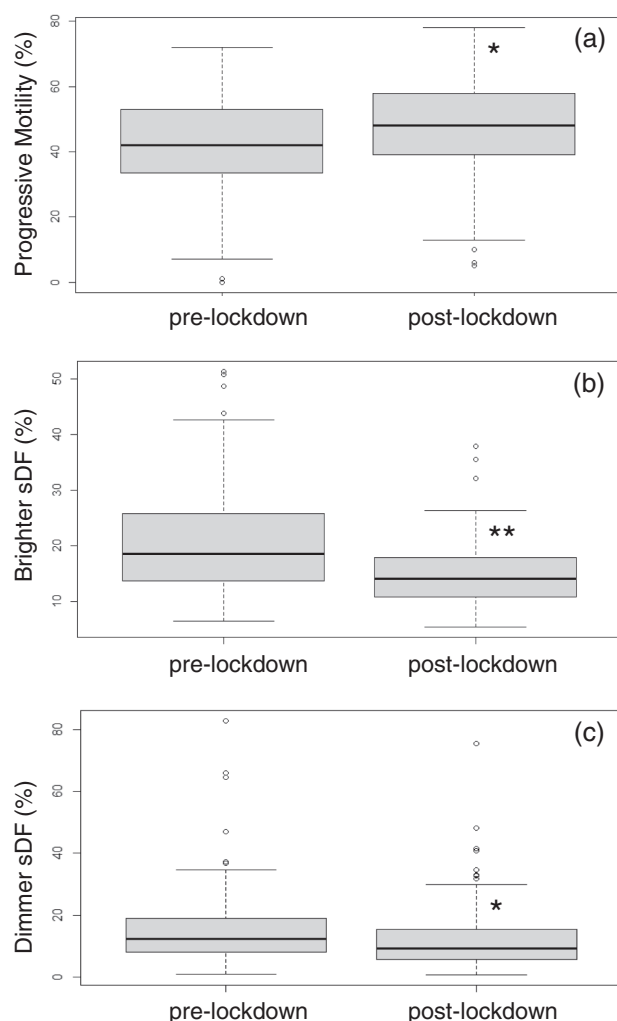
## 2.5 | Statistical analyses

Variable normal distribution was checked by Kolmogorov–Smirnov test. Variables with a non-normal distribution were expressed as median [IQR]. Categorical variables were expressed as percentages. The Pearson's Chi-squared test and the Wilcoxon test were used to assess whether there were differences between the two groups (pre- and post-lockdown). In particular, Pearson's Chi-squared test with Yates' continuity correction was used for lifestyle factors and anamnestic data, whereas Wilcoxon rank sum test with continuity correction was used for age and abstinence. The latter test was also used to assess the possible impact of lockdown on outcomes (total, brighter, and dimmer sDF, semen volume, semen pH, sperm progressive motility, sperm morphology, sperm concentration, sperm count, and leucocyte concentration). The impact of the lockdown period on sDF fractions, progressive motility, sperm concentration and semen pH were also studied in a first linear regression model introducing

**TABLE 1**
 Age, abstinence, lifestyle factors, occupational exposure to toxicants and/or high temperature, drug and supplements assumption, and anamnestic data of patients recruited in pre- ( $n = 119$ ) and post-lockdown ( $n = 105$ ).

Variable		Pre-lockdown	Post-lockdown	p-value
Age (y)		38.00 [34.00–44.00]	38.00 [33.00–42.50]	.398 <sup>b</sup>
Abstinence (day)		4.00 [3.00–4.50]	4.00 [3.00–4.50]	.777 <sup>b</sup>
Sedentary time	<8 h/day	85 (71.4%)	74 (70.5%)	.993 <sup>c</sup>
	>8 h/day	34 (28.6%)	31 (29.5%)	
Exposure to toxicants <sup>a</sup>	Yes	7 (5.9%)	5 (4.8%)	.941 <sup>c</sup>
	No	112 (94.1%)	100 (95.2%)	
Exposure to high temperature	Yes	5 (4.2%)	5 (4.8%)	1.000 <sup>c</sup>
	No	114 (95.8%)	100 (95.2%)	
Smoking habit	Yes	35 (29.4%)	34 (32.4%)	.317 <sup>c</sup>
	No	84 (70.6%)	71 (67.6%)	
Alcohol assumption	No	89 (74.8%)	78 (74.3%)	.938 <sup>c</sup>
	≤2 drinks/day	27 (22.7%)	25 (23.8%)	
	>2 drinks/day	3 (2.5%)	2 (1.9%)	
Supplement assumption	Yes	25 (21%)	17 (16.2%)	.453 <sup>c</sup>
	No	94 (79%)	88 (83.8%)	
Physical exercise	No	42 (35.3%)	42 (40.0%)	.543 <sup>c</sup>
	1–3 h/weekly	52 (43.7%)	36 (34.3%)	
	4–6 h/weekly	19 (16.0%)	21 (20.0%)	
	≥7 h/weekly	6 (5.0%)	6 (5.7%)	
Cryptorchidism	Yes	2 (1.7%)	4 (3.8%)	.569 <sup>c</sup>
	No	117 (98.3%)	101 (96.2%)	
Mumps	No	70 (58.8%)	68 (64.8%)	.634 <sup>c</sup>
	Pre-P	43 (36.1%)	34 (32.4%)	
	Post-P with orchitis	5 (4.2%)	3 (2.9%)	
	Post-P without orchitis	1 (0.8%)	0 (0%)	
Varicocele	No	80 (67.2%)	74 (70.5%)	.867 <sup>c</sup>
	Yes	21 (17.6%)	15 (14.3%)	
	Surgically treated	16 (13.4%)	15 (14.3%)	
	Relapsed	2 (1.7%)	1 (1.0%)	
Inguinal hernia/testicular torsione	Yes	11 (9.2%)	11 (10.5%)	.933 <sup>c</sup>
	No	108 (90.8%)	94 (89.5%)	
Urogenital infections	Yes	12 (10.1%)	6 (5.7%)	.340 <sup>c</sup>
	No	107 (89.9%)	99 (94.3%)	
Medications	Yes	30 (25.2%)	30 (28.6%)	.678 <sup>c</sup>
	No	89 (74.8%)	75 (71.4%)	
Disease	Yes	31 (26.1%)	28 (26.7%)	1.000 <sup>c</sup>
	No	88 (73.9%)	77 (73.3%)	
Previous chemo-/radiotherapy	Yes	15 (12.6%)	17 (16.2%)	.566 <sup>c</sup>
	No	104 (87.4%)	88 (83.8%)	

Note: Data are median [IQR]. For categorical variables, both number of patients and percentage are presented.  
 Abbreviation: P, puberty.  
<sup>a</sup>Pesticides, organic solvents, petroleum derivatives, Pb, Cd, radiation.  
<sup>b</sup>Wilcoxon rank sum test.  
<sup>c</sup>Pearson's Chi-squared test.



**FIGURE 2** Progressive motility (a), brighter (b), and dimmer (c) sDF. Box graphs report median values (interquartile range) of the indicated parameters. \*\* $p < .001$ ; \* $p < .01$ . #, Wilcoxon rank sum test.

leukocyte concentration as covariate and in a second linear regression model introducing also abstinence, age and several conditions known to affect sperm progressive motility and/or sDF as covariates. To compare air pollutant levels between 2019 and 2020, and between exposure of the pre- and post-lockdown groups, we used the Mann-Whitney  $U$  test. A  $p$ -value  $< .05$  was considered statistically significant. Analyses were performed using statistical software R and Statistical Package for the Social Sciences (SPSS 26) for Windows (SPSS, Chicago, IL) (4.2.0). SPSS 26 and Microsoft PowerPoint were used for artwork.

### 3 | RESULTS

To evaluate the effect of lockdown on sDF levels, we recruited 119 and 105 men among subjects undergoing routine semen analysis and sDF determination in our center before and after lockdown, respectively. To evidence differences in aspects possibly affecting

sDF, we compared the two groups in terms of age, abstinence, lifestyle factors, occupational exposure to toxicants and/or high temperature, drug and supplements assumption and anamnestic data (Table 1). As shown, no difference was observed between the two groups.

The two groups were compared also regarding semen parameters. We found that progressive motility was higher in the post-lockdown group (Figure 2a), which also exhibited a lower concentration of leukocytes (Table 2). We also observed a decrease in sperm concentration, albeit not affecting sperm count, and an increase in semen pH (Table 2). All the other tested variables showed no difference between the two groups (Table 2). We then evaluated whether there was a difference in sDF level, between the two groups. We found that both brighter ( $p < .001$ ) and dimmer sDF ( $p < .01$ ) were lower in the post- than pre-lockdown group (Figure 2b,c, respectively). Consequently, total sDF showed a clear decrease after lockdown (24.79 [18.33–33.97]% vs. 35.02[25.04–45.73]%,  $p < .001$ ).

Since leukocyte concentration was found to be lower after lockdown, the impact of the lockdown period on sperm progressive motility, sperm concentration, semen pH, and sDF was studied also in a linear regression model introducing leukocyte concentration as covariate. Results indicated that leukocyte concentration did not affect either the increase of progressive motility or of semen pH ( $p < .05$  and  $p < .001$ , respectively) or the decrease of brighter and dimmer sDF ( $p < .001$  and  $p < .01$ , respectively), observed after lockdown. Conversely, the model considering leukocyte concentration blunted the difference in sperm concentration between the pre and post-lockdown group ( $p > .05$ ). To further assess whether other possible confounders affected the observed impacts of lockdown on the main sperm parameters, we used a second linear regression model, adjusting also for all the relevant conditions known to affect sperm parameters and sperm DNA quality. Results of this analysis (Table 3) indicate the improvement of sperm motility but not sperm concentration. Regarding sDF, the model confirmed the decrease in both brighter and dimmer sperm populations, and consequently of total sDF. The model also unveiled some well-known relationships. Indeed, both total (Lu et al., 2020; Vaughan et al., 2020) and dimmer sDF (Muratori, Marchiani, et al., 2015) were positively associated with age, while sperm motility and concentration were negatively associated with exposure to high temperature (Garolla et al., 2013). A negative correlation was also reported between sperm concentration and sedentary lifestyle (Gaskins et al., 2015; Priskorn et al., 2016). Unexpectedly, in our study dimmer sDF was negatively correlated with consumption of less than 2 drinks/day whereas no significant relationship was observed in heavier drinkers consuming more than 2 drinks/day (Table 3). However, this finding maybe related to lower power of analysis for the latter group ( $N = 5$  out of 224 total participants). The impact of lockdown was studied also distinguishing smokers ( $N = 69$ ) and nonsmokers ( $N = 155$ ). Results are reported in Table 4, indicating a decrease of both total and brighter sDF after lockdown in both groups, whereas the decrease of dimmer sDF did not reach the statistical significance (smoker:  $p = .120$ ; nonsmoker:  $p = .148$ ). Further, after lockdown, only in nonsmokers we observed changes similar to those observed in all subjects, i.e. the improvement of progressive



**TABLE 2** Routine semen parameters in patient groups before and after lockdown.

Variable	Pre-lockdown (n = 119)	Post-lockdown (n = 105)	p-value <sup>a</sup>
Volume (mL)	3.30 [2.30–4.40]	3.70 [2.60–4.70]	.182
pH	7.60 [7.60–7.80]	7.80 [7.60–7.80]	.001
Sperm concentration (×10 <sup>6</sup> /mL)	51.35 [22.50–90.00]	35.25 [18.30–66.75]	.043
Sperm counts (×10 <sup>6</sup> /ejaculate)	143.10 [62.16–340.00]	115.00 [72.42–238.10]	.124
Progressive motility (%)	42.00 [33.00–53.00]	48.00 [38.50–58.00]	.007
Total motility (%)	54.00 [48.00–60.00]	57.00 [49.00–64.00]	.093
Morphology (%)	4.00 [2.0–6.00]	4.00 [2.0–6.00]	.559
Leukocytes (×10 <sup>6</sup> /mL)	0.00 [0.00–0.20]	0.00 [0.00–0.00]	.007

Note: Data are median [IQR].

<sup>a</sup>Wilcoxon rank sum test.

motility, the decrease of leukocyte concentration and the increase of pH (Table 4). Conversely, similar values for these parameters were found in post- and pre- lockdown groups of smokers (Table 4).

## 4 | DISCUSSION

In this study, we compared sDF levels and routine semen parameters between men referred to infertility clinics before and after a lockdown period from March to May/June in 2020, when most activities and movement of peoples were highly restricted. We found an increase in sperm progressive motility and a decrease in sDF levels and leukocytes concentration. In addition, an increase in semen pH, albeit remaining within a physiological range, was observed post lockdown. No other semen parameter resulted changed.

The increase of sperm progressive motility and the decrease of sDF after lockdown appear in agreement with the reported negative relationship between motility and sDF (Cheng et al., 2020; Le et al., 2019). In addition, the concomitant decrease in leukocyte concentration is consistent with the observed negative effect of leukocyte concentration on both progressive motility (Lobascio et al., 2015) and sDF (Lobascio et al., 2015; Mahfouz et al., 2010).

Interestingly, no change was found between the two groups regarding conditions which could affect sDF amount and/or progressive motility, including exposure to toxicant (Irnandi et al., 2021; Recio-Vega et al., 2008) or high temperature (Garolla et al., 2013), smoking habits (Axelsson et al., 2022; Sharma et al., 2016), alcohol consumption (Amor et al., 2022; Martini et al., 2004), varicocele (Blumer et al., 2008; Pallotti et al., 2018), and recent urogenital infections (Pagliuca et al., 2021; Weidner et al., 2013). The two groups were also similar for age, abstinence, drug consumption, daily sedentary time, and intensity of physical exercise (Table 1), all affecting sperm progressive motility and/or DNA quality as well (Gaskins et al., 2015; Hallak et al., 2020; Lu et al., 2020; Priskorn et al., 2016; Vaughan et al., 2020). Hence, none of these factors appears to be responsible for the changes in sperm parameters observed by the study. In the model taking into account all these

possible confounders, patients analyzed after lockdown still showed an increase of progressive motility and a decrease of sDF fractions (Table 3).

The impact of lockdown on sDF was also observed when we studied smokers and nonsmokers separately, albeit the decrease of this sperm damage was seen only in total and brighter fractions. Indeed, although a trend toward lower values of dimmer sDF after lockdown was observed both in smokers and in nonsmokers, the decrease did not reach the statistical significance (Table 4). Regarding the other sperm parameters, only in nonsmokers we saw the improvement of progressive motility, the decrease of leukocyte concentration and the increase of pH. Conversely, smokers did not show these effects. These results suggest that smoking could counteract the benefit associated with lockdown. However, it is also possible that the lack of statistical significance was due to the smaller sample size for this analysis in smokers ( $N = 69$  smokers and  $N = 155$  nonsmokers).

As mentioned, none of the conditions known to affect sperm motility/DNA quality seem to be involved in the improvement of these sperm traits after lockdown. Among alternative possible causes, we considered the sharp decrease in air pollution which occurred during lockdown as reported by many studies (Uday et al., 2022; Wu et al., 2022). As expected, also in Tuscany area, where the recruited subjects lived, such decrease occurred, as published by the local environmental protection agency ([https://www.arpat.toscana.it/temi-ambientali/aria/qualita-aria/grafici\\_bollettino](https://www.arpat.toscana.it/temi-ambientali/aria/qualita-aria/grafici_bollettino)). Among monitored air pollutants ( $\mu\text{g}/\text{m}^3$ ),  $\text{C}_6\text{H}_6$  (0.52[0.41–0.77] vs. 0.76[0.60–1.00],  $p < .001$ ),  $\text{SO}_2$  (1.07[0.77–1.47] vs. 2.22[1.79–3.00],  $p < .001$ ),  $\text{NO}_2$  (24.03 [18.03–31.45] vs. 37.18[31.37–46.19],  $p < .001$ ),  $\text{CO}$  (0.36 [0.31–0.40] vs. 0.43[0.37–0.54],  $p < .001$ ) resulted as highly reduced comparing the same period (March 9–June 11) in 2020 and 2019, respectively. In 2020, also  $\text{H}_2\text{S}$  resulted decreased, albeit not reaching statistical significance ( $p = .063$ ). In addition, the seasonal variation in air pollution (Chen et al., 2020), further supports a higher exposure of the pre- versus post-lockdown group. Indeed, with the only exception of  $\text{O}_3$ , all air pollutants monitored in the Tuscany area showed higher concentrations in the June 2019–February 2020 than in the March 2020–November 2020 period (to account for the duration of

**TABLE 3** Linear regression model of the association between sperm concentration, sperm progressive motility and total, brighter, and dimmer sDF with the indicated variables.

Coefficient estimate (SE)	Concentration	p-value	Progressive motility	p-value	Total sDF	p-value	Brighetr sDF	p-value	Dimmer sDF	p-value
Pre/post lockdown	−0.23 (0.15)	.119	0.054 (0.03)	.046	−0.28 (0.05)	.000	−0.30 (0.06)	.000	−0.25(0.10)	.012
Age	−0.00 (0.01)	.638	−0.00 (0.00)	.062	0.01 (0.00)	.008	0.00 (0.00)	.503	0.02 (0.01)	.001
Abstinence	0.09 (0.05)	.075	−0.01 (0.01)	.246	0.03 (0.02)	.061	0.03 (0.02)	.147	0.04 (0.03)	.156
Leukocyte concentration	0.17 (0.18)	.340	0.01 (0.03)	.832	−0.12 (0.07)	.075	−0.12 (0.07)	.067	−0.08 (0.12)	.495
Sedentary time (>8 h/day)	−0.39 (0.17)	.021	−0.05 (0.03)	.072	0.11 (0.06)	.96	0.01 (0.06)	.885	0.20 (0.11)	.070
Exposure to toxicants	0.29 (0.34)	.389	0.05 (0.06)	.413	−0.20 (0.13)	.131	−0.12 (0.13)	.356	−0.23 (0.22)	.296
Exposure to high temperature	−1.04 (0.36)	.004	−0.14 (0.07)	.040	−0.00 (0.14)	.997	−0.25 (0.14)	.066	0.26 (0.24)	.263
Smoking habit	0.18 (0.10)	.074	0.02 (0.02)	.242	−0.07 (0.04)	.090	−0.07 (0.04)	.0605	−0.06 (0.06)	.353
Alcohol <sup>a</sup> 1–2 drinks/day	−0.09 (0.018)	.632	−0.00 (0.03)	.886	−0.04 (0.07)	.614	0.06 (0.07)	.392	−0.24 (0.12)	.047
Alcohol <sup>a</sup> >2 drinks/day	0.61 (0.53)	.253	0.09 (0.10)	.338	0.09 (0.21)	.666	0.25 (0.20)	.219	−0.25 (0.35)	.463
Physical exercise <sup>b</sup> 1–3 h/week	−0.05 (0.17)	.767	−0.04 (0.03)	.236	0.05 (0.07)	.461	0.00 (0.07)	.950	0.09 (0.11)	.405
Physical exercise <sup>b</sup> 4–6 h/week	−0.14 (0.21)	.519	0.00 (0.04)	.933	−0.01 (0.08)	.910	0.00 (0.08)	.967	−0.02 (0.14)	.873
Physical exercise <sup>b</sup> ≥7 h/week	0.16 (0.34)	.639	0.08 (0.06)	.175	−0.11 (0.13)	.418	−0.12 (0.13)	.354	−0.24 (0.22)	.278
Varicocele <sup>c</sup>	−0.33 (0.22)	.123	−0.03 (0.04)	.392	0.00 (0.08)	.975	−0.09 (0.08)	.257	0.15 (0.14)	.295
Surgically treated varicocele <sup>c</sup>	−0.36 (0.21)	.082	−0.04 (0.04)	.343	0.00 (0.08)	.968	0.00 (0.08)	.959	0.07 (0.13)	.623
Relapsed varicocele <sup>c</sup>	−0.23 (0.69)	.737	−0.04 (0.13)	.772	0.46 (0.27)	.087	0.56 (0.26)	.034	0.43 (0.45)	.337
Urogenital infections	0.18 (0.28)	.532	−0.05 (0.05)	.330	0.08 (0.11)	.474	0.10 (0.11)	.346	−0.03 (0.18)	.884
Medications	−0.08 (0.17)	.621	0.02 (0.03)	.530	0.02 (0.07)	.782	−0.01 (0.06)	.837	0.07 (0.11)	.527

<sup>a</sup>Reference category: No drink.<sup>b</sup>Reference category: No physical exercise.<sup>c</sup>Reference category: No varicocele.

spermatogenesis, we included at least 90 days before semen collection in each group; Table S1 and Figure S1).

To support the role of air pollutant exposure, many mechanisms have been proposed by both in vitro and animal models, explaining how these compounds could affect both sperm motility and DNA integrity. Airborne particulate particles can bind trace elements of noxious compounds such as polycyclic aromatic hydrocarbons (PAHs). In mice, it has been reported that PAHs can act as endocrine disruptors, disturbing the hypothalamic–pituitary axis, decreasing testosterone levels, and impairing sperm motility (Jeng & Yu, 2008). The effect on sperm motility provoked by particulate matter was also found in humans by Hammoud et al., who suggested that the impairment of motility was caused by the alteration of the synthesis of late proteins involved in motility development (Hammoud et al., 2010). Further, the increase of sDF observed in men exposed to high levels of particulate matter was attributed to the ability of reactive metabolites of PAHs to form adducts with DNA of spermatids and epididymal spermatozoa, where DNA repair machinery is no longer available (Rubes et al.,

2005). Finally, in a rodent model, exposure to PM<sub>2.5</sub> resulted in the increase of reactive oxygen species (ROS) production and induction of apoptotic pathways, ultimately leading to sperm DNA damage (Zhang et al., 2018). It has also been hypothesized that, similarly to cigarette smoking (Saleh et al., 2002; Vine et al., 1994), air pollutants could induce seminal ROS production and leukocytes infiltration (Sokol et al., 2006), both promoting a condition where ROS exceed natural antioxidant defenses, that is, oxidative stress. The role of leukocytes in mediating the effect of air pollution on sperm function appears consistent with the decrease in the semen concentration, accompanying the improvement of sperm motility and DNA quality, observed in our study after lockdown period. In addition, oxidative stress is a well-known cause of loss of both sperm motility (Yu & Huang, 2015) and DNA integrity (Aitken et al., 2016). Indeed, ROS attack promotes lipid peroxidation, which in turn is responsible for changes in membrane fluidity and integrity and thus in sperm motility (Aitken et al., 2010). In addition, ROS attack is one of the mechanisms originating sDF, acting especially during transit in the male genital tract (Muratori,

**TABLE 4** Routine semen parameters in smokers and nonsmokers before and after lockdown.

Variable	Smokers			Nonsmokers		
	Pre-lockdown (n = 35)	Post-lockdown (n = 34)	p-value <sup>a</sup>	Pre-lockdown (n = 84)	Post-lockdown (n = 71)	p-value <sup>b</sup>
Age (y)	39.00 [34.00–44.00]	38.00 [33.00–43.25]	.543	38.00 [34.00–44.00]	37.00 [34.00–42.00]	.599
Volume (mL)	3.30 [2.10–4.20]	3.60 [2.50–4.80]	.534	3.25 [2.40–4.47]	3.60 [2.50–4.80]	.493
Total sDF	36.25 [24.12–46.06]	21.79 [14.51–26.59]	.011	34.41 [25.11–45.25]	25.69 [20.45–39.18]	.004
Brighter sDF	17.40 [12.53–25.84]	13.17 [9.29–16.35]	.011	18.74 [14.47–25.77]	14.80 [11.11–20.44]	.000
Dimmer sDF	13.10 [8.80–19.82]	8.71 [4.39–11.46]	.120	12.11 [7.25–18.93]	9.90 [5.99–17.27]	.148
pH	7.60 [7.60–7.80]	7.80 [7.60–7.85]	.094	7.60 [7.60–7.80]	7.60 [7.60–7.80]	.024
Sperm concentration (×10 <sup>6</sup> /mL)	55.00 [23.00–90.00]	41.88 [21.58–84.63]	.258	49.25 [22.13–89.50]	33.00 [15.00–59.50]	.034
Sperm count (×10 <sup>6</sup> /ejaculate)	129.15 [62.16–358.55]	150.88 [78.63–276.19]	.183	144.77 [62.63–298.95]	101.85 [48.75–215.02]	.072
Progressive motility (%)	44.00 [34.00–55.00]	51.00 [39.75–59.50]	.132	42.00 [32.25–52.00]	47.00 [36.00–57.00]	.037
Total motility (%)	54.00 [47.00–64.00]	59.50 [52.00–66.25]	.189	54.00 [49.00–59.00]	55.00 [47.00–64.00]	.353
Morphology (%)	4.00 [3.00–6.00]	5.00 [3.00–7.00]	.645	4.00 [2.00–6.00]	4.00 [2.00–5.00]	.110
Leukocytes (×10 <sup>6</sup> /mL)	0.00 [0.00–0.10]	0.00 [0.00–0.00]	.242	0.00 [0.00–0.28]	0.00 [0.00–0.00]	.005

Note: Data are median [IQR].

<sup>a</sup>Smokers.

<sup>b</sup>Nonsmokers.

<sup>a,b</sup>Wilcoxon rank sum test.

Tamburrino, et al., 2015). Interestingly, we found that the decrease of total sDF observed after lockdown was due to a decrease in DNA damage of both brighter and dimmer sperm populations (Muratori et al., 2008). Likely, the two sperm populations have a different site and mechanism of origin of sDF (Lotti et al., 2017; Muratori, Tamburrino, et al., 2015). Indeed in dimmer spermatozoa, sDF appears to be mainly caused by testis apoptosis (Muratori, Tamburrino, et al., 2015), whereas in brighter sperm population, sDF is more likely to be linked to oxidative attack occurring during the transit in male genital tract (Muratori, Tamburrino, et al., 2015). Indeed, not only is dimmer sDF associated to a very high caspase activity (Marchiani et al., 2014), but its amount is also well correlated with signs of testis impairment (Lotti et al., 2017). On the other hand, only in brighter viable spermatozoa sDF is associated to oxidative damage to DNA and membrane (Muratori, Tamburrino, et al., 2015) and only brighter sDF correlates with signs of inflammation of prostate and genital tracts (Lotti et al., 2017). Hence, it is suggested that both apoptosis and oxidative attack are involved in the higher values of sDF found in the pre-lockdown group, in agreement with the mentioned involvement of these two mechanisms in induction of sperm DNA damage by air pollution, observed in a rodent model (Zhang et al., 2018).

In conclusion, although we cannot draw a sharp cause/effect relationship, we believe that a lower exposure to air pollution is the most probable cause of the improvement in sperm motility and DNA quality observed in men after the lockdown period of between March and May/June 2020. Indeed, a lower exposure to air pollutants occurred in the post-lockdown group, and no difference was observed between the pre- and post-lockdown group in conditions known to affect levels of sperm motility and sDF.

However, the study has several limitations. First, we used individual measures of sperm parameters but aggregated levels of pollutants, hence we do not know whether recruited subjects did experience a decreased exposure to air pollution. Second, as the stay-at-home order deeply changed most people's lives, we cannot rule out that other confounders, different from those assessed, had a role in improving sperm motility and DNA quality of spermatozoa in men after lockdown. For instance, during lockdown a change in patterns and habits of diet has been reported (Bennett et al., 2021) and could have an effect of sperm functions (Salas-Huetos et al., 2017). Third, the study was based on men seeking infertility care and, as such, conclusions may not apply to general male population. Finally, the cross-sectional study design did not allow the comparison of the same subjects before and after lockdown, that would minimize inter-individual variability. Keeping in mind these limitations, the study, however, suggests a possible role of air pollution exposure in the development of poor sperm motility and DNA quality and thus in male infertility.

# AUTHOR CONTRIBUTIONS

Costanza Calamai collected data on air pollution and participated in statistical analyses, graph building and writing the manuscript. Oumaima Ammar collected data on routine semen analysis, sDF determination, and those coming from the questionnaire. Sara Marchiani performed sDF determination. Selena Degl'innocenti, Marisa Fino, and Sara Dabizzi performed routine semen analysis. Lorenzo Righi performed statistical analyses. Mario Maggi, Elisabetta Baldi, and Linda Vignozzi critically discussed the results. Monica Muratori conceived, designed the study and drafted the manuscript. All authors critically reviewed the manuscript and gave their approval.



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## CONFLICT OF INTEREST STATEMENT

The authors have no relevant financial or nonfinancial interests to disclose.

## DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## CONSENT FOR PUBLICATION

All Authors and the responsible authorities at the institute where the work has been carried out gave their consent to publish the article.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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