Exposure to polycyclic aromatic hydrocarbons and nicotine, and associations with sperm DNA fragmentation

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Abstract

Background: Tobacco smoking has been reported to cause DNA fragmentation and has been suggested to cause mutations in spermatozoa. These effects have been ascribed to the action of polycyclic aromatic hydrocarbons (PAH) present in the smoke. Simultaneously, DNA fragmentation has been associated with mutagenesis.

Objective: The aim of this study was to investigate whether levels of urinary biomarkers of PAH and nicotine exposure were associated with sperm DNA fragmentation.

Methods: In the urine of 381 men recruited from two cohorts of young men (17–21 years old) from the general Swedish population, the PAH metabolites 1-hydroxypyrene and 2-hydroxyphenanthrene, as well as the nicotine metabolite cotinine, were measured. The sperm DNA fragmentation index (DFI) was analysed using the sperm chromatin structure assay. Associations between the DFI, and PAH metabolite levels as continuous variables as well as in quartiles, were studied by general linear models adjusted for abstinence time. A similar analysis was carried out for cotinine levels, according to which the men were categorised as “non-smoking” (n = 216) and “smoking” (n = 165).

Results: No association was found between levels of any of the three biomarkers and DFI, either as a continuous variable (p = 0.87–0.99), or when comparing the lowest and the highest quartiles (p = 0.11–0.61). The same was true for comparison of men categorised as non-smoking or smoking (DFI 11.1% vs. 11.8%, p = 0.31).

Discussion: We found no evidence of PAH or nicotine exposure to be associated with DFI, which does not exclude that these exposures may have other effects on sperm DNA.

Conclusion: In these young men, levels of biomarkers of nicotine and PAH exposure were not associated with DFI.

KEYWORDS
biomarkers, cotinine, mutagen exposure, PAH, smoking, sperm DNA fragmentation

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1 INTRODUCTION

A common exposure in humans – smoking – is associated with reductions in several parameters of semen quality, a but has also been suggested to cause cancer and malformations in children due to mutations in paternal germ cells exerted by mutagens in tobacco smoke. Those mutagens include certain polycyclic aromatic hydrocarbons (PAH) that can bind to the DNA and thus cause DNA adducts, which in turn can lead to mutations in the DNA sequence. Another type of DNA damage that can be caused by compounds in both tobacco smoke and air pollution is DNA strand breakage. This kind of breakage seems to have a potential to lead to mutations due to errors occurring during the DNA repair. Thus, one could expect that a positive association between exposure to PAH or smoking and sperm DNA fragmentation would point to chemicals in cigarette smoke as possible causes of mutations in the spermatozoa and potentially also in the offspring. Although active smoking is a major source of PAH, exposure to PAH may also originate from exposure to environmental tobacco smoke, diet and air pollution, albeit seemingly at lower levels. So, to fully capture the total PAH exposure, levels of biomarkers in humans were suggested to represent a more appropriate estimate of the total exposure to PAH, than information on smoking habits only.

Previously, some studies reported that levels of PAH metabolites were associated with sperm DNA fragmentation but other studies did not find an association. Furthermore, the results appear to be incongruent regarding the type of PAH metabolite for which such an association was found.

Nonetheless, the pyrene metabolite 1-hydroxypyrene (1-OHP) measured in urine has been reported to be associated with smoking in a dose-related manner, and has also been reported as a useful biomarker for PAH exposure from other sources at least in non-smokers. Similarly, as for 1-OHP, 2-hydroxyphenanthrene (2-OHPH), which is a metabolite of phenanthrene, has been reported to be associated with smoking and has also, when measured in pregnant women, been associated with a shorter telomere length in children as a marker of DNA damage.

Despite seemingly good evidence that smoking does induce strand breaks in sperm, we were previously unable to find an association between self-reported smoking and sperm DNA fragmentation assessed by the sperm chromatin structure assay (SCSA). Still, self-reported data on smoking seem to underestimate the true smoking, whereas measuring the nicotine metabolite cotinine in biological fluids has been suggested as the most appropriate indicator of tobacco smoke exposure. Thus, cotinine levels quantify the body’s internal dose of nicotine and have been reported to be more strongly associated with health outcomes as compared to the reported number of cigarettes smoked per day. In addition, an association between levels of cotinine and sperm DNA fragmentation has been reported.

For the assessment of sperm DNA fragmentation, SCSA is one of the most commonly used tests and seems to have good precision and repeatability. DFI as determined by SCSA is associated with the risk of infertility, independent of other parameters of semen quality. Furthermore, SCSA has been suggested to be a suitable method to study DNA fragmentation due to chemical exposure that is mutagenic to male germ cells. This suggestion is supported by studies reporting that mutagenic exposure increases sperm DNA fragmentation according to both SCSA and the TUNEL assay, similar to the increase seen by SCSA after tobacco smoke exposure, which as mentioned has also been suggested to be mutagenic to human germ cells and reported to give rise to mutations in both sperm and spermatogonial stem cells of mice. Accordingly, the aim of this study was to investigate whether urinary levels of 1-OHP, 2-OHPH and cotinine, as biomarkers of exposure to PAH and nicotine, were associated with sperm DNA fragmentation in Swedish men from the general population.

2 METHODS

2.1 Participants

In 2000–2001, 305 men between the ages 18 and 21 years and from the general population were recruited to the Reproductive Medicine Centre in Malmö, Sweden, through the medical health examination at the National Service Administration in Sweden (NSAS) prior to military service, like described by Richthoff et al. Similarly, in 2008–2010, we recruited 314 men between the ages 17 and 20 years and also from the general population living within 60 km from the city of Malmö. The latter group of men was recruited both through the medical health examination at NSAS prior to a possible military service (N = 241) and through announcements at schools (N = 73), like described by Axelsson et al. All of the men, 619 in total, signed an informed consent, answered questionnaires regarding their use of tobacco, delivered samples of urine and semen on the same day and were paid about 50 euros for their participation. The data collection and study of associations between exposure and reproductive function was approved by the local ethical review board at Lund University, and was in line with the Declaration of Helsinki. Regarding ethnicity, 73% of the men recruited during 2000–2001 were born and raised in Sweden and had mothers born and raised in Sweden, whereas only men fulfilling these two criteria were asked to participate in the recruitment that took place during 2008–2010.

Out of the 619 men, we had information available about (1) the time of abstinence from ejaculation before the delivery of the semen sample in 601 men; (2) levels of the sperm DNA fragmentation index (DFI) in 573 men and (3) levels of 1-OHP, 2-OHP and cotinine in the urine of 400 men (which was the number of men from whom urinary samples were available after other analyses had been performed). For 381 men, data on both abstinence time, levels of DFI and of the exposure markers were available. Among the 573 men with available values of DFI, the DFI values were statistically significantly lower in the 381 men for whom we had data on abstinence time and levels of 1-OHP, 2-OHPH and cotinine, as compared to those 192 for whom these data were missing (DFI 14% vs. 16%, p = 0.04). Among the 381 men on...
whom we had all of the above data, data on self-reported tobacco use and long-term/chronic diseases (the most common of which was allergy or asthma [seven men]) are shown in Table 1. Fifty-seven men smoked 1–9 cigarettes per day, and 32 men smoked 10 cigarettes or more per day. The highest amount of cigarettes smoked per day was 20 (reported by one participant). The self-reported data on tobacco use included the use of “snus” (sometimes referred to as [moist oral] snuff), an oral tobacco used under the upper lip. The mean age of the men was 18 years (standard deviation [SD] 0.4 years) and the median age was 18 years as well, ranging from 17 to 21 years.

2.2 | Determination of sperm DNA fragmentation

All the 381 men delivered samples of semen which were analysed by SCSA. This assay indicates the proportion of spermatozoa with breaks in the DNA, as previously described for the two cohorts. In brief, adding of acridine orange to the samples, which gives a red fluorescence when interacting specifically with single-stranded DNA in sperm cells with a supposed DNA fragmentation, enables the proportion of spermatozoa with a red emission (spermatozoa with sperm DNA damage/strand breaks) to be measured by use of flow cytometry of 5000–10,000 sperm cells, as described in detail by Bungum et al. The SCSA was performed in the same way, without changes in methodology in 2000–2001 and 2008–2010.

2.3 | Analyses of the biomarkers

In the urine of the included men, levels of 1-OHP and 2-OHPH were analysed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Details of the method have been described by Alhamdow et al. The limit of detection of these two compounds was 50 pg/ml, whereas the coefficients of variation in duplicate urinary samples were 14% for 1-OHP at 0.5 ng/ml, and 9% for 2-OHPH at 0.8 ng/ml. Urinary cotinine was analysed using a modified method for serum in which the limit of detection was 0.4 ng/ml and the coefficient of variation 2% at 48 ng/ml. In brief, for the three compounds, urine samples were hydrolysed using β-glucuronidase (Escherichia coli) and incubated for 30 min at 37°C. Deuterium-labelled internal standards were used in the analysis. The samples were analysed using a QTRAP5500 (AB Sciex, Framingham, MA, USA). All analyses were performed at the Department of Occupational and Environmental Medicine at Lund University. The methods used for analyses of 1-OHP and cotinine are part of a quality control programme between analytical laboratories coordinated by the University of Erlangen-Nuremberg, Germany. The laboratory also participates in the ICI/EQUAS exercises for the analysis of 1-OHP and 2-OHPH and is approved by the HBM4EU project.

The mean value of urinary cotinine in the 288 self-reported non-smokers was 690 ng/ml or 370 µg/g creatinine, and the geometric mean was 13 ng/ml or 7.7 µg/g creatinine. The corresponding mean values in the smokers were 2300 ng/ml or 1400 µg/g creatinine, and the geometric mean was 40 ng/ml or 890 µg/g creatinine.

2.4 | Statistical analyses

Due to a skewed distribution of values, DFI was transformed by the natural logarithm. We considered p < 0.05 as statistically significant, and studied associations between DFI and the levels of cotinine as well as the two PAH metabolites as continuous variables, by using univariate general linear models in SPSS and having both abstinence time and one metabolite at a time as covariates in the model. For the analysis that included cotinine, we also separately adjusted for the use of snus (yes/no). Thereafter, we studied main effects using type II sum of squares, as we did not expect abstinence time to interact with PAH metabolite levels regarding associations with DFI. For the levels of cotinine, we also calculated the Pearson product-moment correlation coefficient with the levels of DFI.

Further, we additionally divided metabolite levels in quartiles and studied whether the men in the highest quartile had a higher DFI than the men in the lowest quartile; again adjusting for abstinence time with an additional separate analysis for cotinine by adjusting also for the use of snus. Also for the analysis of categorised exposure levels, we applied linear regression models (general linear models).

Subsequently, for all three metabolites, the above-mentioned analyses were repeated by adjusting for the urinary concentration of creatinine (mmol/L). Based on urinary cotinine levels, the participants were categorised into smokers and non-smokers with the former group defined as those with values above 100 µg/g creatinine. Using this cut-off, 165 men were categorised as smokers and 216 as non-smokers.

Subsequently, we studied the difference in DFI between the men categorised as smokers and non-smokers adjusting for abstinence time, and subsequently even after adjusting for the use of snus (yes/no). The group of men categorised as smokers (based on the levels of cotinine) also included those who used snuff but not cigarettes, and despite adjusting for the use of snuff, we also compared self-reported smokers with self-reported non-smokers regarding DFI in this larger group of men than in our previous study, again by adjusting for abstinence time. In addition to this, we separately used the number of cigarettes smoked per day, adjusted for abstinence time, and studied the association with DFI. We also separately studied whether a difference could be found between men who reported that they smoked 10 cigarettes per day or more (n = 32) and those who smoked less than 10

| TABLE 1 | Background characteristics on tobacco use and disease among the 381 included men |
|---------|-----------------|-----------------|-----------------|
|         | Yes             | No              | Missing         |
|         | N   | %       | N   | %       | N   | %       |
| Self-reported smokinga | 89  | 23%    | 288 | 76%    | 4   | 1%      |
| Use of “snus”b | 66  | 17%    | 279 | 73%    | 36  | 9.4%    |
| Long-term/chronic disease | 18  | 4.7%   | 359 | 94%    | 4   | 1.0%    |

a Missing values due to smoking other than cigarettes.
b Oral tobacco used under the upper lip.
TABLE 2  Levels of exposure, DNA fragmentation index (DFI) and abstinence time of the 381 included men

| Variable | Unit | Mean ± standard deviation | Median (range) |
|----------|------|---------------------------|----------------|
| 1-OHP    | ng/ml urine | 0.17 ± 0.29 | 0.11 (<LOD–4.5) |
|          | ng/mmol creatinine | 11 ± 16 | 7.0 (0.16–240) |
| 2-OHPH   | ng/ml urine | 0.35 ± 0.44 | 0.23 (0.053–5.9) |
|          | ng/mmol creatinine | 25 ± 43 | 15 (3.0–580) |
| Cotinine | ng/ml urine | 1100 ± 1700 | 36 (<LOD–9600) |
|          | ng/mmol creatinine | 6900 ± 120,000 | 2600 (<LOD–860,000) |
| DFI      | % | 14 ± 11 | 11 (2.9–87) |
| Abstinence time | Hours | 69 ± 47 | 60 (8–500) |

Abbreviations: 1-OHP, 1-hydroxypyrene; 2-OHPH, 2-hydroxyphenanthrene; DFI, DNA fragmentation index.

TABLE 3  Mean levels of the different compounds in the highest and lowest quartile

| Biomarker | Unit | Lowest quartile | N | Highest quartile | N |
|-----------|------|-----------------|---|-----------------|---|
| 1-OHP     | ng/ml urine | <LOD | 97 | 0.40 | 95 |
|          | µg/g creatinine | <LOD | 95 | 0.20 | 95 |
| 2-OHPH    | ng/ml urine | 0.11 | 94 | 0.78 | 97 |
|          | µg/g creatinine | 0.099 | 95 | 0.49 | 95 |
| Cotinine  | ng/ml urine | 0.59 | 95 | 3600 | 95 |
|          | µg/g creatinine | 0.41 | 95 | 1900 | 95 |

Abbreviations: 1-OHP, 1-hydroxypyrene; 2-OHPH, 2-hydroxyphenanthrene.

cigarettes per day (n = 349), with same adjustment. A similar comparison was made between men smoking 10 cigarettes per day or more (n = 32) and men who reported that they did not smoke at all (n = 292).

In a post-hoc power analysis (powerandsamplesize.com), if we calculated with a power (1 – β) of 0.80, a type I error rate (α) of 5%, as well as with a DFI of 10.7% in the lowest quartile and a standard deviation in DFI of 11%, then for comparing 96 men in the highest quartile of exposure to 1-OHP with 96 men in the lowest quartile of exposure, we would need a 37% higher DFI (a DFI of 14.7%, which is still a clinically normal value52) in the highest quartile of exposure to find a statistically significant difference.

3  RESULTS

Background characteristics on exposure levels, DFI and abstinence time are shown in Table 2, and mean levels of exposure markers in the lowest and highest quartiles of the different exposure markers are shown in Table 3.

3.1  Exposures as continuous variables and DFI

None of the associations between the two urinary exposure markers for PAH (1-OHP [shown in Figure 1] and 2-OHPH) or the urinary biomarker for nicotine exposure, cotinine (all expressed per millilitre urine), and DFI was statistically significant (p = 0.87–0.99, Table 4).

The same was true for the associations after adjusting the analysis regarding cotinine for the use of snus (p = 0.94), and the urinary levels of the compounds for the concentration of creatinine in the urine (p = 0.62–0.94, Table 4). No statistically significant correlation was found between the levels of cotinine and DFI (Pearson correlation −0.065, p = 0.20).

FIGURE 1  Scatter/dot graph showing how DNA fragmentation index (DFI) related to the urinary levels of 1-OHP in the 381 men
TABLE 4  Regression coefficients for the different biomarkers of exposure from general linear models, with DNA fragmentation index (DFI) (%) transformed by the natural logarithm as a dependent variable, adjusted for abstinence time

|                      | Regression coefficient, $\beta$ (95% confidence interval) | $p$ Value |
|----------------------|-----------------------------------------------------------|-----------|
| 1-OHP                | -0.017 (-0.23 to 0.20)                                     | 0.87      |
| 1-OHP<sup>a</sup>    | 0.99 (-2.9 to 4.9)                                         | 0.62      |
| 2-OHPH<sup>b</sup>   | 0.01 (-0.14 to 0.14)                                       | 0.99      |
| 2-OHPH<sup>c</sup>   | 0.070 (-1.4 to 1.5)                                        | 0.92      |
| Cotinine<sup>a</sup>| -0.000017 (-0.000053 to 0.000019)                          | 0.35      |
| Cotinine<sup>b</sup>| -0.000017 (-0.000045 to 0.000042)                          | 0.94      |
| Cotinine<sup>c</sup>| -0.000002 (-0.000045 to 0.000042)                          | 0.94      |

<sup>a</sup>Levels adjusted also for the urinary concentration of creatinine.

<sup>b</sup>Adjusted also for the use of oral tobacco snus (yes/no), $n = 345$.

TABLE 5  Levels of DFI (back-transformed) according to the quartile of exposure, adjusted for abstinence time

| DFI          | Lowest quartile (%) | Highest quartile (%) | Ratio between the lowest and highest quartile | $p$-value for difference |
|--------------|---------------------|----------------------|-----------------------------------------------|--------------------------|
| 1-OHP        | 10.7                | 11.2                 | 0.96                                          | 0.61                     |
| 1-OHP<sup>a</sup> | 11.4                | 11.5                 | 0.99                                          | 0.84                     |
| 2-OHPH       | 10.7                | 12.3                 | 0.87                                          | 0.11                     |
| 2-OHPH<sup>b</sup> | 10.3                | 11.9                 | 0.87                                          | 0.11                     |
| Cotinine     | 10.9                | 11.4                 | 0.96                                          | 0.64                     |
| Cotinine<sup>a</sup>| 11.3                | 11.3                 | 1.00                                          | 0.98                     |
| Cotinine<sup>b</sup>| 10.4                | 11.0                 | 0.95                                          | 0.57                     |
| Cotinine<sup>c</sup>| 10.8                | 11.1                 | 0.97                                          | 0.76                     |

<sup>a</sup>Levels adjusted also for the urinary concentration of creatinine.

<sup>b</sup>Adjusted also for the use of the oral tobacco called “snus” (yes/no), $n = 345$.

3.2  | Exposures as categorised variables and DFI

When comparing the highest and lowest exposure quartiles of the three different exposure markers in relation to the levels of DFI, no statistically significant difference in DFI was seen for 1-OHP, 2-OHPH or cotinine ($p = 0.11–0.64$, Table 5). These results remained not statistically significant also after adjusting the urinary levels of these compounds for the urinary concentration of creatinine ($p = 0.11–0.98$), and regarding cotinine for the additional adjustment for the use of snus (Table 5).

3.3  | Smokers versus non-smokers and DFI

No statistically significant difference was found in DFI between men who, based on their urinary concentration of the nicotine metabolite cotinine, were categorised as non-smokers and those who were categorised as smokers (11.1% vs. 11.8%, $p = 0.31$). After adjusting for the use of the oral tobacco “snus”, again no statistically significant difference was found between the non-smokers and the smokers (10.2% vs. 11.1%, $p = 0.26$).

When comparing self-reported smokers with self-reported non-smokers, we found no statistically significant difference in DFI between the smokers and the non-smokers (11.1% vs. 11.2%, $p = 0.84$). In addition, no statistically significant association was found between the number of cigarettes smoked per day (as a continuous variable) and DFI ($p = 0.28$), nor between men smoking 10 or more cigarettes per day and men smoking less (12.6% vs. 11.3%, $p = 0.34$) or not at all (12.6% vs. 11.2%, $p = 0.32$).

4  | DISCUSSION

In this study of young Swedish smoking and non-smoking men from the general population, we did not find that levels of the urinary biomarkers for PAH exposure, 1-OHP and 2-OHPH, or for nicotine were associated with DFI according to the SCSA. Thus, in our cohort exposures to pyrene, phenanthrene and nicotine seemed not to be main contributors to fragmentation of sperm DNA as assessed by SCSA. We also found no difference in DFI between self-reported smokers and non-smokers, nor between those smoking more than 10 cigarettes per day and those smoking less, or not at all.

Our results are in line with our previous study, which was based on one of the two cohorts included here, in which we did not find an association between self-reported smoking and DFI. Moreover, our results seem to agree with a lack of an association in a majority (seven) of studies using SCSA, included in a review article that covered studies on exposure to tobacco smoke and sperm DNA damage, as well as with some additional studies. Our results are also in line with those of another study in which no association was found between 1-OHP and sperm DNA fragmentation by use of the Comet assay, although the authors found associations for a metabolite of another PAH, fluorene.
Nevertheless, our results differ with those of several other studies that have reported associations between smoking or tobacco use and an increased SCSA-determined DFI. Reasons to why these studies found associations unlike those in our study may be factors such as a high power through having many included men or a high intensity of smoking – since a higher DFI was reported specifically for heavy-smoking men. An additional possible explanation could be investigation of specifically infertile men who may be more sensitive to tobacco smoke-induced sperm DNA fragmentation, for example due to a potentially less condensed DNA which seems to be more common in infertile men. A less condensed DNA could increase the risk of sperm DNA damage due to agents present in tobacco smoke. Furthermore, regarding the lack of an association between levels of biomarkers for PAH exposure and DFI in our study, our results seem to differ to the only previous study that addressed these associations in men who, like in our study, were not exposed to high levels of PAH through occupational exposure. Those authors reported an association between 1-OHP and DFI (although the information on the level of statistical significance was not presented, and no association was found between smoking and DFI). A possible reason for the fact that an association was found between 1-OHP and DFI in that study but not in ours may be because their study was about double the size of ours. Potentially, ethnic or dietary differences between China and Sweden could also explain the difference.

Although it may be difficult to compare PAH exposure in our study with PAH exposure in studies of occupationally exposed men, the lack of an association between levels of PAH biomarkers in our study stands in contrast to an association reported between levels of 1-OHP and DFI in coke-oven workers, although another study on exposed workers did not find a statistically significantly higher DFI in workers with higher 1-OHP exposure than in controls. An explanation for why we did not find an association between urinary levels of 1-OHP and DFI, whereas such an association was found in one of the studies, could be that the 1-OHP levels in the men in our study were several orders of magnitude lower.

Regarding studies that used other methods to assess sperm DNA fragmentation, our results seem to differ from the majority of studies regarding smoking, which reported associations with sperm DNA fragmentation. Our results also differ from an association reported between cotinine and sperm DNA fragmentation in one study. Moreover, our results also seem to differ with those of a study from a polluted area in China in which an association between levels of 1-OHP adjusted for smoking, was associated with indications of sperm DNA damage according to the percentage of tail DNA in spermatozoa found by the Comet assay, although no association was found between 1-OHP and two other parameters of sperm DNA fragmentation: tail length and tail distributed moment. A potential explanation for the difference in the results in our study and these studies could be that, some methods, for unknown reasons, can more easily identify DNA damage caused specifically by smoking or PAH exposure as compared to SCsA.

A strength of our study is the use of cotinine in addition to self-reported smoking as a marker for tobacco smoke exposure, given the stronger association with health outcomes reported for cotinine as compared to self-reported data. Similarly, monitoring of PAH exposure through biomarkers in body fluids has been suggested as the best way to assess the exposure that is taken up by the human body. Our study had the power to find a DFI level about 37% higher in men in the highest exposure quartile versus men in the lowest quartile of any of the three metabolites. This may be compared with a reported 80% higher DFI level in smoking workers that were highly exposed to 1-OHP than in smoking workers who were less exposed to 1-OHP. We have applied SCsA for measurement of DFI as this method has proved valuable in clinical assessment of males from infertile couples, but as mentioned, other methods to assess sperm DNA integrity may be superior to SCsA in detecting PAH or tobacco smoke-related alterations in sperm DNA. The men included in this study were all younger than 22 years, which might be a limitation of the study in view of the higher DFI levels in older individuals, who cannot be excluded to be more susceptible to sperm DNA fragmentation due to chemical exposures.

Overall, we were not able to find associations between either self-reported smoking or the levels of biomarkers of exposure to two PAHs or nicotine (both of which are related to smoking and potential mediators of effects), and sperm DNA fragmentation by use of the SCsA in young Swedish men from the general population. This does not exclude the possibility that tobacco smoking or PAH exposure in men may cause diseases in their children through other mechanisms than sperm DNA fragmentation as assessed by the SCsA.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Jonatan Axelsson designed the study and wrote the draft of the manuscript. Christian H. Lindh was responsible for the analysis of the biomarkers, and described the analytical methods. Professor Aleksander Giwercman provided financing for research time for Jonatan Axelsson and was responsible for the recruitment of study.
participants as well as for the frame of the research project in which the men were recruited. All authors approved the final manuscript.

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