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human reproduction **ORIGINAL ARTICLE** ***Reproductive epidemiology***

Indices of methylation in sperm DNA from fertile men differ between distinct geographical regions

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STUDY QUESTION: Which are the main determinants, if any, of sperm DNA methylation levels?

SUMMARY ANSWER: Geographical region resulted associated with the sperm methylation status assessed on genome-wide repetitive sequences.

WHAT IS KNOWN ALREADY: DNA methylation level, assessed on repetitive sequences from peripheral blood lymphocyte, can vary with age, gender, alcohol consumption and white blood cell counts.

STUDY DESIGN, SIZE, DURATION: A cross-sectional study. Individual data were collected from 269 young healthy men of proven fertility living in three geographical regions: Inuits from Greenland, Caucasians from Warsaw (Poland) and Kharkiv (Ukraine). Semen samples were collected between May 2002 and February 2004 and aliquots were immediately frozen.

PARTICIPANTS/MATERIALS, SETTING, METHODS: We estimated sperm DNA global methylation level (DGML) in two ways. First DNA methylation in repetitive DNA sequences (LINE-1, Satα and Alu) was quantified by PCR pyrosequencing after bisulfite conversion and second by flow cytometry (FCM) using fluorescently labeled monoclonal antibodies anti-5-methylcytosine. We analyzed whether personal characteristics and habits, body mass index, semen quality parameters, sperm chromatin integrity, biomarkers of accessory gland function and the plasma concentration of reproductive hormones were associated with sperm DNA methylation levels in men. Associations were evaluated by analysis of variance and linear regression analyses.

MAIN RESULTS AND THE ROLE OF CHANCE: The geographical location emerged as the main determinant when using the methylation level in repetitive sequences. FCM DGML results were not associated with those from repetitive sequence analysis. No other consistent associations between methylation markers and the assessed variables were identified across countries.

LIMITATIONS, REASONS FOR CAUTION: The methods used are only surrogates of the actual sperm methylome and the methylation levels at individual specific loci were not explored.

WIDER IMPLICATIONS OF THE FINDINGS: Sperm DGML is relatively independent from semen quality parameters and is a new candidate biomarker for epidemiological studies of the impact of environmental contaminants on male fertility.

STUDY FUNDING/COMPETING INTEREST(S): The study is part of the project CLEAR (Climate change, Environmental contaminants and Reproductive health) supported by the European Commission 7th framework program, contract no: FP7-ENV-2008-1-226217. No competing interest is declared.

Key words: human spermatozoa / sperm DNA methylation / pyrosequencing / flow cytometry / epidemiology

Introduction

DNA methylation is a post-replicative, reversible and heritable covalent chemical modification that occurs at the C5 position of the cytosine pyrimidine ring primarily when in a CpG context. DNA methylation is an essential epigenetic mechanism involved in the regulation of a wide array of biological processes, including parental imprinting, tissue-specific gene expression and genomic stability (Seisenberger *et al.*, 2013).

During a man's lifetime, there are three main epigenomic reprogramming periods, each of which involves waves of DNA demethylation and methylation. The first occurs in the preimplantation embryo, the second in primordial germ cells and the third begins at the onset of spermatogenesis. Sperm are the terminally differentiated male germ cells and their methylation patterns represent the zenith of the whole process (Carrell, 2012). During spermatogenesis, most DNA methylation is acquired by the type A spermatogonia but changes occur up to and including the pachytene spermatocyte phase. Subsequently, sperm DNA methylation remains stable (Trasler, 2009).

There is increasing evidence that DNA methylation may be influenced by environmental and lifestyle factors (Alegría-Torres *et al.*, 2011; Faulk and Dolinoy, 2011; Furrow *et al.*, 2011; Feil and Fraga, 2012; Hou *et al.*, 2012). Most of the studies have been carried out with DNA extracted from somatic cells, and it remains unknown how vulnerable the epigenome reprogramming during spermatogenesis is in the adult. This information might be important in the ongoing quest for environment- and lifestyle-related risk factors that could undermine human male fertility (Sharpe, 2010). In this respect, recent studies have demonstrated that diet (Lambrot *et al.*, 2013) and chemicals with endocrine active properties can disrupt male germ cell epigenomic reprogramming in rodents (Daxinger and Whitelaw, 2012; Guerrero-Bosagna and Skinner, 2012).

It has been estimated that more than one-third of DNA methylation occurs in retrotransposonic repetitive elements: due to their high occurrence throughout the genome and their heavy methylation level. Alu and LINE-1 have been proposed as surrogate markers for estimating indirectly DNA global methylation level (DGML) (Yang *et al.*, 2004). Among the variety of methods assessing DNA methylation, pyrosequencing after sodium bisulfite selective conversion of cytosine to uracil represents a precise and cost-effective technique to map 5-methyl cytosine (5-mC) at a single nucleotide resolution level, requiring relatively small amounts of genomic DNA (Tost and Gut, 2007). Furthermore, immunostaining of 5-mCs has also been proposed as an alternative (non-sequence specific) approach for assessing DGML (Benchaib *et al.*, 2003, 2005). Sperm DNA global methylation was shown to be independent from semen quality, sperm DNA fragmentation and relative abundance of protamines (Benchaib *et al.*, 2003; Aoki *et al.*, 2006), whereas it was strongly associated with fertilization rate and embryo quality following *in vitro* fertilization (Benchaib *et al.*, 2005).

Because of the growing interest in genome-wide assessment of DNA methylation for epidemiological studies, knowledge on whether and how DNA methylation levels vary as a function of individuals' characteristics is of relevance to make epidemiological surveys more complete and effective. Determinants of the sperm methylation level in adult healthy fertile individuals are still unknown. In order to investigate the role of potential confounding factors on the sperm global methylation level, we studied the impact of demographic and lifestyle factors, and of hormonal and seminal correlates in a population of proven fertile men from three geographic areas (Greenland, Ukraine, Poland), enrolled in the seventh framework EU-funded project CLEAR (Climate change, Environmental contaminants and Reproductive health).

Materials and Methods

Semen samples

A detailed description of the procedures for the enrollment and inclusion of the subjects can be found elsewhere (Spanò *et al.*, 2005; Bonde *et al.*, 2008). Briefly, semen samples were collected between May 2002 and February 2004 from Greenlandic Inuits, and from the general population in Warsaw, Poland and Kharkiv, Ukraine. Descriptive parameters of semen quality and sperm chromatin integrity, together with reproductive hormone plasma concentrations and seminal levels of biomarkers for epididymal and accessory gland function, were collected within the framework of a previous EU-funded project (INUENDO, Biopersistent organochlorines in diet and human fertility) (Bonde *et al.*, 2008). The samples were analyzed for concentration, motility and morphology according to a protocol specifically defined for the project based on the latest version (at the time of sample collection) of the World Health Organization (WHO, 1999) guidelines (Toft *et al.*, 2006). From the frozen samples stored in the INUENDO Biobank, 116 from Greenland, 100 from Warsaw and 100 from Kharkiv were randomly selected and included in this study.

The study was approved by the local ethical committees representing all the participating populations and all subjects signed an informed consent.

Sperm DNA global methylation analysis

We assessed the sperm global methylation level by flow cytometric (FCM) immunodetection of 5-mC. We have independently assessed the methylation level of the retrotransposonic sequences Alu and LINE-1 as a proxy of the sperm global methylation level. To further increase genomic coverage (Bollati *et al.*, 2009, 2011; Fabris *et al.*, 2011), we additionally analyzed the methylation status of Satα, a 171-bp long satellite tandem DNA repeat, representing the main DNA component of every human centromere (Casa and Gabellini, 2012).

Flow cytometry immunodetection of 5-mC: the FCM sperm DNA global methylation assay

FCM immunodetection of 5-mC was done by published methods (Benchaib *et al.*, 2005; Barzideh *et al.*, 2013) with some modification. The complete protocol is provided in Supplementary data. The primary antibody was a mouse IgG1 monoclonal antibody, anti-5-methylcytidine (5-mC) (Serotec, Clone 33D3, Oxford, UK) coupled with AlexaFluor488-conjugated Fab anti-mouse IgG1 fragments, using the Zenon technology (Invitrogen, S. Giuliano Milanese, Italy) according to the manufacturer's instructions. After 60 min incubation at room temperature in the dark, samples were counterstained with 5 μg/ml propidium iodide (Sigma, St. Louis, Mo, USA). Cells (10 000 events) were analyzed by an FACScalibur (Becton Dickinson, San José, CA, USA). Off-line analysis of the FCM data was carried out by the FlowJo software (Tree Star, Inc., Ashland, OR, USA). The mean, median and standard deviation of the resulting green fluorescence intensity distribution were calculated and expressed as channel number of the fluorescence acquisition scale.

Two investigators, blind to all the information regarding the samples except their code, performed the FCM analyses. Forty seven of the 316 samples could not be measured because of an insufficient cell number at the end of manipulation procedures.

At the end, we measured 269 samples, 75 from Greenland, 97 from Warsaw (Poland) and 97 from the Kharkiv district (Ukraine).

Alu, LINE-1 and Satα methylation assays

For these assays, DNA was extracted from a subgroup of samples undergoing the FCM analysis by a commercial kit (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany). In total, 500 ng of DNA were treated using EpiTect Bisulfite kit (Qiagen) according to the manufacturer's protocol. The analyses of Alu and Satα elements were conducted according to previously published methods (Yang *et al.*, 2004; Bollati *et al.*, 2007, 2009). The detailed protocol is described in Supplementary data. Primer sequences and fragment lengths are listed in Supplementary data, Table SI. LINE-1 methylation level was evaluated with PyroMark CpG LINE-1 kit (Qiagen). Pyrosequencing was carried out using the PyroMark ID Q96 system (Biotage AB, Uppsala, Sweden) according to manufacturer's instructions. Methylation level quantification was performed using the provided software. The percentage of methylation (%5-mC) was expressed for each DNA locus as the number of 5-mC divided by the sum of methylated and unmethylated cytosines. One investigator, blind to all the information regarding the samples except their code, performed the pyrosequencing analyses.

LINE-1 analysis was done in 224 samples, 90 from Greenland, 65 from Warsaw (Poland) and 69 from the Kharkiv district (Ukraine). For the Satα analysis, we collected data from 213 samples, 81 from Greenland, 63 from Warsaw and 69 from the Kharkiv district. Alu methylation was evaluated on 215 samples, 84 from Greenland, 64 from Warsaw and 67 from the Kharkiv district.

Statistical analysis

General linear models (SAS GLM procedure) were used to analyze crude and adjusted associations of demographic, seminal and hormonal characteristics with the sperm DNA global methylation parameters. Multiple regression models were used to evaluate associations between Alu, LINE-1 and Satα methylation levels (expressed as %5-mC) together with the average fluorescence intensity from FCM measurements (expressed as channel number) and subjects' characteristics, i.e. country (Greenland, Warsaw, Kharkiv), age, body mass index (BMI kg/m^2 continuous), smoking (blood cotinine level), alcohol drinking (drinks/week) and abstinence time (ln transformed abstinence time in days). We also assessed the associations between methylation level and semen quality parameters, i.e. sperm concentration (ln transformed millions/ml), normal morphology (% normal sperm), motility [% progressive motile sperm, class a + b (WHO, 1999)] and sperm chromatin structure parameters assessed by the SCSA (Evenson *et al.*, 2002), i.e. DNA fragmentation index (%DFI) and high DNA stainable (%HDS) cells, as well as associations between seminal biochemical parameters of epididymal and sex accessory gland function, i.e. neutral α-glucosidase, fructose, zinc and prostate-specific antigen (PSA) and reproductive hormone concentration, i.e. testosterone, sex hormone-binding globulin (SHBG), estradiol, follicle-stimulating hormone (FSH), luteinizing hormone (LH) and inhibin B plasma levels. In models for male reproductive function, we adjusted for male age, smoking, alcohol and abstinence time. Statistical analyses were performed using the SAS statistical software 1 version 9.1 (SAS Institute, Inc., Cary, NC, USA).

Results

Characteristics of the study population

The characteristics of the study populations are reported in Table I. Mean and standard errors are provided for each parameter, for each geographical location (Greenland, Kharkiv, Warsaw) and for the overall population (Combined). The distributions of sperm DNA global methylation parameters approximated to a normal (Gaussian) pattern and are reported as mean ± SEM)

Sperm DGML end-points

As far as the FCM DGML is concerned, the average value (expressed as channel numbers of the fluorescence intensity) for all the study population was 251 ± 7.6. The highest level of global methylation (273 ± 13.8) was observed in Kharkiv and the lowest (234 ± 14.0) was reported for the Greenland Inuits.

The methylation levels (%5-mC) obtained after pyrosequencing the different regions were as follows: (i) LINE-1, overall mean 77 ± 0.4%, the highest value being in Greenland (79 ± 0.4%), followed by Kharkiv (77 ± 0.9%) and Warsaw (76 ± 0.4%); (ii) Alu, the overall mean 23 ± 0.3%, the highest value being in Kharkiv (24 ± 0.4%) followed by Warsaw (23 ± 0.5%) and the lowest in Greenland (21 ± 0.4%); (iii) Satα, overall mean 47 ± 0.9%, the highest value being in Greenland (51 ± 1.5%) and the lowest in Warsaw (42 ± 1.3%).

Associations of methylation parameters as a function of demographic and lifestyle factors

FCM DGML

Abstinence time was the only characteristic statistically significantly associated with FCM DGML, for each unit increase in ln abstinence time there is a change of −43.1 (CI −66.7 to −19.3) units of FCM DGML (Table II). When looking separately at each country, the association between abstinence time and FCM DGML was only statistically significant in Warsaw and no other covariates were associated with FCM DGML at the country level (data not shown). When the results were compared with Warsaw as reference the results of the FCM DGML were not significantly different across the three geographical areas.

LINE-1

In the combined population, smoking status was the only characteristic statistically significantly associated with the LINE-1 methylation level (Table II). The LINE-1 methylation level was 1.5 (CI 0.1–2.9)% higher in smokers compared with non-smokers. LINE-1 data were statistically different in the three countries. In particular, LINE-1 methylation levels were 3.3 (CI 1.6–5.1)% higher in Greenland compared with Warsaw and 2.3 (CI 0.4–4.3)% higher in Kharkiv compared with Warsaw.

Alu

Alu methylation level was not associated with any other covariate either when considering the overall population or considering the three countries separately (Table II). The percentage of Alu methylation in samples from Greenland was statistically significantly 2.7 (CI 1.2–4.2)% lower than in Warsaw, whereas the two European towns were not different.

Satα

No associations were evident for Satα in the combined populations. Satα methylation levels in Greenland were 6.9 (CI 2.1–11.7)% higher than Warsaw, whereas the levels in Warsaw and Kharkiv were similar (Table II).

Associations between sperm DNA global methylation variables and male reproductive function parameters

Sperm concentration, morphology and motility were negatively associated with FCM DGML, whereas the SCSA parameters DFI and HDS were positively associated.

Table I Characteristics (mean ± SEM) of subjects included in the combined analysis of determinants of sperm DNA methylation level.

Variable	Greenland	Kharkiv	Warsaw	Combined
Demographic and lifestyle characteristics				
Age (years)	30.8 ± 0.6 (*N* = 116)	26.4 ± 0.5 (*N* = 99)	30.1 ± 0.4 (*N* = 98)	29.2 ± 0.3 (*N* = 313)
BMI (kg/m^2)	26.5 ± 0.4 (*N* = 114)	24.5 ± 0.3 (*N* = 99)	26.0 ± 0.3 (*N* = 97)	25.7 ± 0.2 (*N* = 310)
Cotinine (ng/ml)	163.4 ± 15.0 (*N* = 112)	156.7 ± 16.7 (*N* = 97)	39.1 ± 9.5 (*N* = 98)	121.6 ± 8.8 (*N* = 307)
Alcohol (drinks/week)	8.4 ± 1.1 (*N* = 114)	2.8 ± 0.2 (*N* = 72)	5.7 ± 0.6 (*N* = 88)	6.1 ± 0.5 (*N* = 274)
Semen quality parameters				
Abstinence time (days)	3.7 ± 0.3 (*N* = 116)	4.1 ± 0.2 (*N* = 99)	6.8 ± 0.6 (*N* = 99)	4.8 ± 0.2 (*N* = 314)
Semen volume (ml)	3.9 ± 0.1 (*N* = 116)	4.3 ± 0.2 (*N* = 99)	4.6 ± 0.2 (*N* = 99)	4.3 ± 0.1 (*N* = 314)
Sperm concentration (× 10^6/ml)	80.8 ± 5.5 (*N* = 116)	83.6 ± 5.9 (*N* = 99)	94.9 ± 8.3 (*N* = 99)	86.1 ± 3.8 (*N* = 314)
Sperm normal morphology (%)	7.1 ± 0.4 (*N* = 115)	7.6 ± 0.4 (*N* = 98)	6.9 ± 0.4 (*N* = 99)	7.2 ± 0.2 (*N* = 312)
Motile sperm (%)	58.1 ± 1.6 (*N* = 115)	58.1 ± 1.9 (*N* = 99)	62.0 ± 1.9 (*N* = 94)	59.3 ± 1.0 (*N* = 308)
Biochemical seminal markers				
NAG (mU/ml)	5.9 ± 0.3 (*N* = 107)	7.1 ± 0.4 (*N* = 97)	8.2 ± 0.4 (*N* = 97)	7.1 ± 0.2 (*N* = 301)
Fructose (mM)	15.7 ± 0.7 (*N* = 111)	14.1 ± 0.6 (*N* = 98)	15.4 ± 0.7 (*N* = 99)	15.1 ± 0.4 (*N* = 308)
PSA (mg/l)	1092.6 ± 63.6 (*N* = 111)	911.5 ± 61.2 (*N* = 98)	1193.3 ± 61.3 (*N* = 99)	1067.4 ± 36.4 (*N* = 308)
Zn (mM)	1.7 ± 0.1 (*N* = 111)	1.7 ± 0.1 (*N* = 98)	2.2 ± 0.1 (*N* = 99)	1.9 ± 0.1 (*N* = 308)
Hormones				
Testosterone (nM)	15.0 ± 0.5 (*N* = 96)	17.9 ± 0.6 (*N* = 97)	12.8 ± 0.4 (*N* = 76)	15.5 ± 0.3 (*N* = 269)
SHBG (nM)	28.6 ± 1.0 (*N* = 96)	28.1 ± 1.0 (*N* = 97)	22.7 ± 1.1 (*N* = 76)	26.8 ± 0.6 (*N* = 269)
Estradiol (pM)	67.3 ± 1.7 (*N* = 96)	82.5 ± 2.6 (*N* = 97)	77.3 ± 4.3 (*N* = 76)	75.6 ± 1.7 (*N* = 269)
FSH (IU/l)	4.5 ± 0.2 (*N* = 96)	4.1 ± 0.2 (*N* = 97)	4.3 ± 0.3 (*N* = 76)	4.3 ± 0.1 (*N* = 269)
LH (IU/l)	4.6 ± 0.2 (*N* = 96)	4.1 ± 0.2 (*N* = 97)	4.2 ± 0.2 (*N* = 76)	4.3 ± 0.1 (*N* = 269)
Inhibin B (ng/l)	191.3 ± 7.14 (*N* = 96)	200.0 ± 6.6 (*N* = 97)	151.4 ± 6.9 (*N* = 76)	183.1 ± 4.2 (*N* = 269)
SCSA parameters				
DFI (%)	8.8 ± 0.5 (*N* = 116)	13.8 ± 0.9 (*N* = 97)	13.2 ± 0.9 (*N* = 99)	11.8 ± 0.4 (*N* = 312)
HDS (%)	13.1 ± 0.8 (*N* = 116)	10.7 ± 0.6 (*N* = 97)	10.0 ± 0.6 (*N* = 99)	11.3 ± 0.4 (*N* = 312)
DNA methylation end-points				
FCM DGML (channel no.)	234.0 ± 14.0 (*N* = 75)	272.6 ± 13.8 (*N* = 97)	242.9 ± 11.6 (*N* = 97)	251.1 ± 7.6 (*N* = 269)
LINE-1, %5-mC	79.2 ± 0.4 (*N* = 90)	76.8 ± 0.9 (*N* = 69)	75.5 ± 0.6 (*N* = 65)	77.4 ± 0.4 (*N* = 224)
Satα, %5-mC	51.3 ± 1.5 (*N* = 81)	44.9 ± 1.6 (*N* = 69)	42.3 ± 1.3 (*N* = 63)	46.6 ± 0.9 (*N* = 213)
Alu, %5-mC	20.8 ± 0.4 (*N* = 84)	24.1 ± 0.4 (*N* = 67)	23.3 ± 0.5 (*N* = 64)	22.6 ± 0.3 (*N* = 215)

Subjects with at least one measurement from the four global methylation parameters were included. DFI, DNA fragmentation index; FCM DGML, flow cytometry sperm DNA global methylation level; HDS, high DNA stainability; NAG, neutral α-glucosidase; PSA, prostate-specific antigen; SCSA, sperm chromatin structure assay; SHBG, sex hormone-binding globulin; 5-mC, 5-methylcytosine.

The methylation levels of LINE-1 and Alu were not associated with any of the semen parameters, whereas the methylation levels of Satα were negatively associated with sperm concentration and with Zn and LH concentration (Table III).

Discussion

This study represents one of the first and largest efforts aimed at investigating possible predictors of human sperm DGML. Among the variables taken into account, only the geographical location (i.e. Greenland versus European towns) showed a statistically significant association with the DNA repetitive sequences' methylation level. Furthermore, for LINE-1, a statistically significant difference emerged also between Warsaw and Kharkiv. However, it should be noted that the pattern of this association was not consistent across the different sequences and no association of the geographical area emerged with FCM DGML. Further studies could clarify to what extent methods of detection and selection of targets may contribute to these inconsistencies. Obviously, the association does not imply a cause–effect relationship, also because the term 'geographical region' includes a number of dietary, environmental and genetic factors not disentangled in our study. Nevertheless, our results showing that geographical area, together with some unknown factors, has an influence on sperm methylation point to an attractive direction for future research.

The epigenetic difference which emerged in this study between Inuit and European populations has some analogy with other differences we observed in the same populations during a previous survey. We reported that Inuits showed, on average, a lower level of SCSA %DFI level

Table II Changes in methylation parameters according to demographic and lifestyle factors (statistically significant associations are shown in bold).

	β-Coefficient (95% CI)			
	FCM DGML	**LINE-1**	**Alu**	**Satα**
Location[a]				
Greenland	−33.5 (−76.1; 9.1)	**3.3 (1.6; 5.1)**	**−2.7 (−4.2; −1.2)**	**6.9 (2.1; 11.7)**
Kharkiv	23.2 (−19.0; 65.3)	**2.3 (0.4; 4.3)**	0.7 (−1.0; 2.4)	2.9 (−2.4; 8.2)
Age (years)	64.5 (−28.0; 157.1)	3.8 (−0.2; 6.9)	0.4 (−2.8; 3.6)	2.0 (−7.7; 11.8)
BMI (kg/m^2)	−22.2 (−144.7; 100.3)	−1.0 (−5.7; 3.8)	−3.3 (−7.5; 1.0)	2.4 (−11.0; 15.7)
Smoking	−5.7 (−40.0; 28.6)	**1.5 (0.1; 2.9)**	−0.1 (−1.2; 1.2)	−2.4 (−6.2; 1.3)
Alcohol	1.3 (−0.8; 3.4)	0.0 (−0.1; 0.1)	0.0 (−0.1; 0.1)	−0.1 (−0.2; 0.2)
Abstinence time	**−43.1 (−66.7; −19.3)**	0.2 (−0.9; 1.3)	−0.4 (−1.4; 0.5)	−1.4 (−4.5; 1.7)

Age, BMI and abstinence time were ln transformed. All associations shown are mutually adjusted for the other covariates in the table. Smoking (yes/no), smokers if cotinine > 10 ng/ml. Alcohol intake in drinks/week (continuous). FCM DGML units are expressed as channel numbers. Units for LINE-1, Alu and Satα are expressed as %5-mC. FCM DGML, flow cytometry sperm DNA global methylation level.
[a]Compared with Warsaw (reference).

Table III Associations between sperm DNA global methylation variables and the parameters of male reproductive function (statistically significant associations are shown in bold).

	β-Coefficient (95% CI)			
	FCM DGML	**LINE-1**	**Alu**	**Satα**
Sperm concentration[a] (×10^6/ml)	**−0.001 (−0.0022; −0.0005)**	0.003 (−0.020; 0.026)	−0.004 (−0.031; 0.023)	**−0.010 (−0.018; −0.002)**
Normal morphology (%)	**−0.00546 (−0.00995; −0.00096)**	0.023 (−0.105; 0.151)	−0.10 (−0.26; 0.05)	0.01 (−0.05; 0.04)
Motile sperm (%)	**0.034 (0.053; 0.0015)**	−0.226 (−0.332; 0.285)	0.45 (−0.11; 0.20)	0.066 (−0.146; 0.279)
NAG (mU/ml)	−0.002 (−0.006; 0.002)	0.038 (−0.072; 0.148)	0.002 (−0.135; 0.139)	−0.040 (−0.082; 0.001)
Fructose (mM)	−0.003 (−0.010; 0.005)	−0.160 (−0.384; 0.064)	0.18 (−0.09; 0.45)	−0.08 (−0.17; 0.01)
PSA (mg/l)	−0.044 (−0.755; 0.667)	0.38 (−19.31; 20.06)	3.58 (−20.34; 27.50)	−7.29 (−14.95; 0.36)
Zn (mM)	−0.004 (−0.002; 0.001)	−0.004 (−0.039; 0.030)	0.02 (−0.02; 0.06)	**−0.02 (−0.03; −0.003)**
Testosterone (nM)	0.002 (−0.004; 0.008)	−0.04 (−0.21; 0.12)	0.09 (−0.11; 0.29)	−0.05 (−0.11; 0.01)
SHBG (nM)	0.003 (−0.009; 0.014)	0.22 (−0.09; 0.53)	−0.25 (−0.62; 0.13)	−0.06 (−0.18; 0.05)
Estradiol (pM)	−0.006 (−0.041; 0.030)	−0.22 (−1.03; 0.59)	0.16 (−0.84; 1.16)	−0.02 (−0.33; 0.29)
FSH (IU/l)	0.0004 (−0.0022; 0.0031)	0.002 (−0.065; 0.069)	−0.05 (−0.13; 0.03)	−0.003 (−0.029; 0.023)
LH (IU/l)	−0.0002 (−0.0022; 0.0018)	0.03 (−0.03; 0.09)	−0.01 (−0.07; 0.06)	**−0.03 (−0.057; −0.013)**
Inhibin (ng/l)	−0.032 (−0.110; 0.045)	1.25 (−1.00; 3.50)	−0.20 (−2.88; 2.48)	−0.65 (−01.50; 0.20)
SCSA DFI (%)	**0.009 (0.001; 0.018)**	−0.07 (−0.30; 0.16)	0.22 (−0.04; 1.25)	−0.04 (−0.13; 0.04)
SCSA HDS (%)	**0.008 (0.001; 0.015)**	−0.12 (−0.35; 0.10)	0.02 (−0.24; 0.29)	0.02 (−0.06; 0.11)

All results are adjusted for age (ln transformed), smoking (>10 ng/ml cotinine); alcohol intake (continuous) and abstinence time (ln transformed). FCM DGML units are expressed as channel numbers. Units for LINE-1, Alu and Satα are expressed as %5-mC. DFI, DNA fragmentation index; FCM DGML, flow cytometry sperm DNA global methylation level; HDS, high DNA stainability; NAG, neutral α-glucosidase; PSA, prostate-specific antigen; SCSA, sperm chromatin structure assay; SHBG, sex hormone-binding globulin.
[a]ln transformed.

compared with the European men (Spanò *et al.*, 2005), a result confirmed also in the population subset considered in this study. Sperm DNA damage differences were also independently corroborated by the results of the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end-labeling) assay (Stronati *et al.*, 2006). Furthermore, as far as the androgen receptor genetic polymorphism is concerned, Inuit men were also characterized, on average, by a lower number of CAG triplets and by a higher frequency of the GGN = 23 allele when compared with European men (Giwercman *et al.*, 2007, 2008). Thus, we can speculate that unexplored environmental exposures, gene–environment interactions or other unmeasured lifestyle or personal factors, such as diet, may influence the expression and activity of DNA methyltransferases

(Denis et al., 2011) and/or availability of methyl groups, playing a role behind the observed differences between Inuit and European men.

A comparison between the repetitive sequence methylation levels we measured in sperm with those reported in peripheral blood lymphocytes (PBLs) by the same technique shows a good overlapping for Alu and LINE-1 (Zhu et al., 2012), whereas, Satα appears to be hypomethylated (Bollati et al., 2009, 2011; Fabris et al., 2011) in agreement with previous reports (Weisenberger et al., 2005; Yamagata et al., 2007; Molaro et al., 2011). Further studies should hopefully clarify the biological meaning of such similarities and differences in relation to cell lineage differentiation and function (Sasaki and Matsui, 2008; Seisenberger et al., 2010; Di Giacomo et al., 2013). Our pyrosequencing data are also in good agreement with other sperm Alu and LINE-1 figures provided by other groups (El Hajj et al., 2011; Kläver et al., 2012).

Human sperm methylomes have started to be deciphered suggesting that sperm is a highly specialized cell type with specific epigenetic signatures (Molaro et al., 2011; Krausz et al., 2012). In sperm of subfertile patients, altered methylation patterns at imprinted and non-imprinted loci have been observed, supporting the notion that defective spermatogenesis can be linked to specific epigenetic derailments in the germ line (Boissonnas et al., 2013). Conversely, inconsistent observations have been collected regarding an association of fertility problems with changes of the sperm global genome methylation level. Benchaib et al. (2005) found that, in a population of infertile patients, the chance to father a child by ART resulted diminished beyond a certain threshold level of anti-5mCs immunofluorescence intensity, a result not confirmed by other authors (Tavalaee et al., 2009). On the other hand, the methylation level of sperm repetitive sequences as a proxy of DGML resulted mostly unchanged in men with fertility problems (Kobayashi et al., 2007; Marques et al., 2008; Boissonnas et al., 2010; Ankolkar et al., 2013). Further studies should be conducted to compare the outputs of different methods measuring sperm methylation patterns in reciprocal relation to each other in order to clarify the influence of the detection method upon the observations and possibly to reconcile the conclusions.

We found that sperm DNA methylation end-points were prevalently unrelated to the parameters of the conventional semen quality assessment. When an association emerged, usually it was neither consistent across the different countries nor across the different sperm global methylation parameters. In our hands, FCM DGML was weakly associated with the SCSA chromatin integrity marker. A negative correlation with sperm DNA fragmentation, evaluated by the TUNEL assay, has been observed for a cohort of infertility patients (Tunc and Tremellen, 2009). FCM DGML resulted also associated with abstinence time. It cannot be excluded that these associations, which are not consistent with the results of the other global methylation end-points, might represent a chance finding due to the multiple statistical tests performed. Therefore, sperm DNA methylation end-points appear unrelated or poorly associated with the parameters related to sperm chromatin integrity, with the biochemical markers of accessory gland function, with the reproductive hormones.

A possible selection bias due to the decision to include only semen samples from the Biobank with at least 8×10^6 cells because of the methodological requirements could be evoked. However, the semen samples were obtained from men with proven fertility, all partners of pregnant women. Consequently, the impact of such a potential bias, if any, is likely to be minor because the frequency of samples not matching the inclusion criteria was small (<9%) and the INUENDO Biobank contains few oligozoospermic samples.

It should not be overlooked that we carried out our methylation analyses on semen samples collected almost 10 years before and stored under deep freezing, thus demonstrating the feasibility of retrospective DNA methylation measurements on archival material. This is in agreement with the results of other PBL-based epidemiological studies reliably performed several years after the initial sample collection (Peluso et al., 2012), and also frozen–thawed semen samples demonstrating that, under standard freezing conditions, 5-mC is quite stable (Kläver et al., 2012).

In conclusion, our study showed that sperm DGML, as assessed by the methylation level of the repeated sequences LINE-1, Alu and Satα as a proxy, is statistically associated with the geographical provenience of samples, pointing to a complex array of variables not disentangled by the covariates we have taken into consideration. Sperm DGML appears unrelated or poorly associated with the parameters of the conventional semen quality assessment, with those related to sperm chromatin integrity, with the biochemical markers of sex accessory gland function, with the reproductive hormones. Considering that sperm methylation profile is quite stable with time (Cortessis et al., 2011; Kläver et al., 2013), it can represent a candidate biomarker to evaluate the effects of potential environmental reprotoxicants.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

M.S., G.T. and J.P.E.B. contributed to the study design, data analysis, conceived the article and generated the first draft of this manuscript. G.L. and C.C. contributed to data collection and production, and commented the manuscript. P.E., T.M. and A.B. contributed to data collection and analysis. B.A.G.J., A.G., V.Z., J.K.L., H.S.P. and D.H. contributed to the data collection, study design and data analysis. All authors revised the manuscript critically and approved the final version of this article.

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Conflict of interest

The authors have no financial interest or other conflicts of interest in the publication of these results.

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