

Altered DNA methylation in estrogen-responsive repetitive sequences of spermatozoa of infertile men with shortened anogenital distance

Christian De Geyter

M.E. Gill, University of Basel, Switzerland

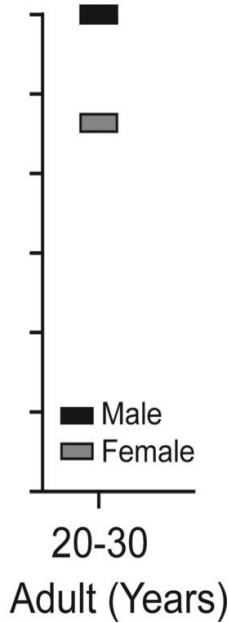
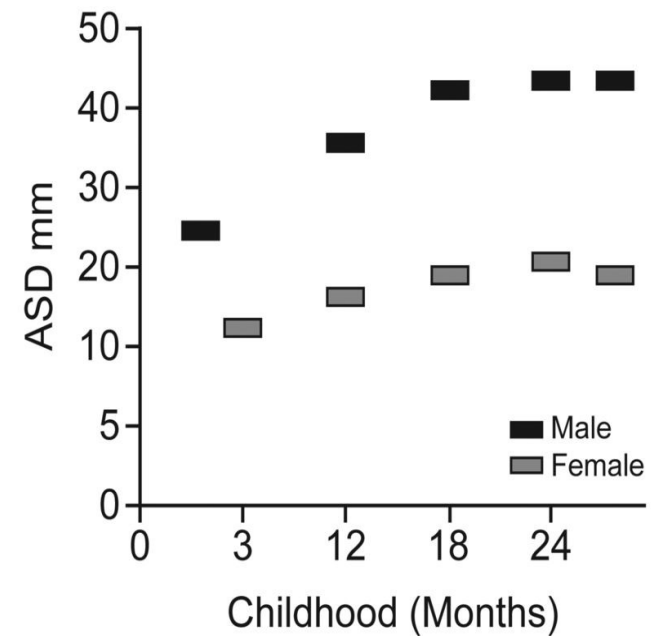
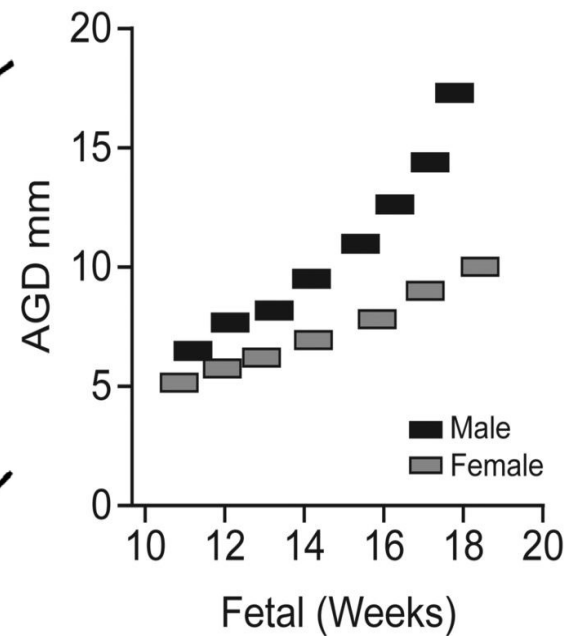
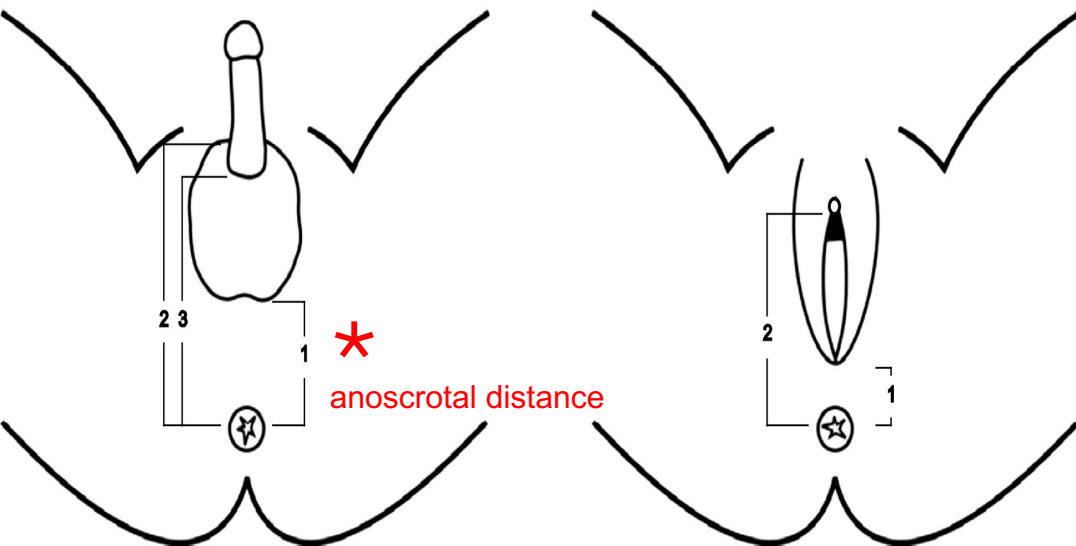
and together with:

L. Stenz, A. Giacobino, University of Geneva, Switzerland

BISC, Global Bioinformatics and Statistics Consulting, Ghent, Belgium

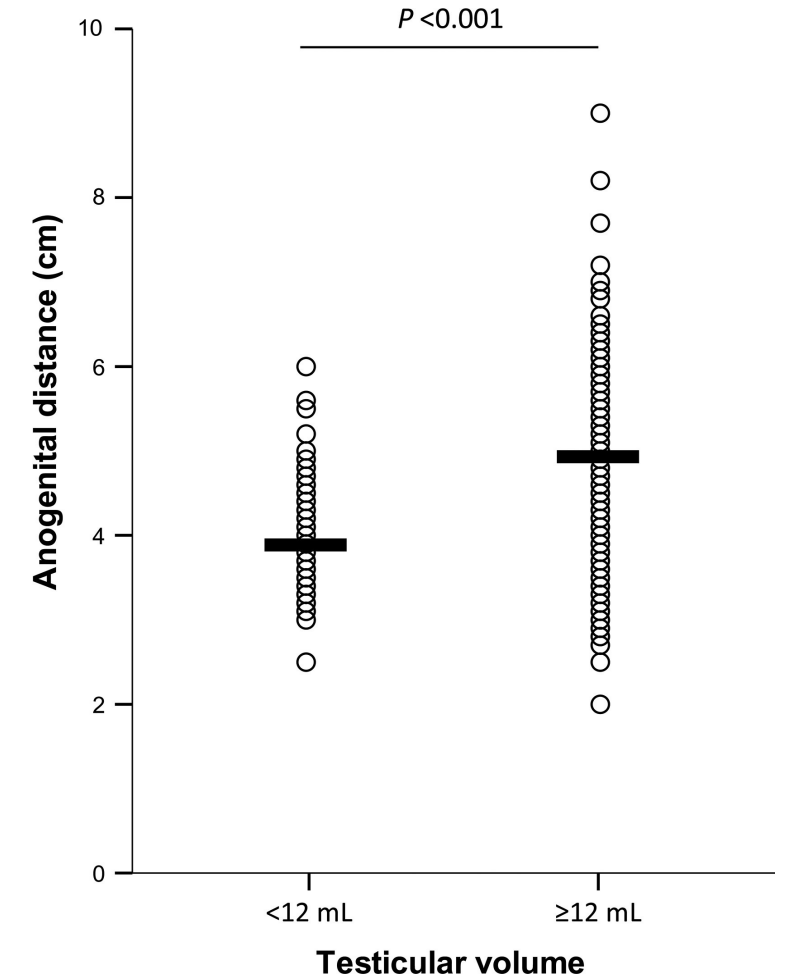
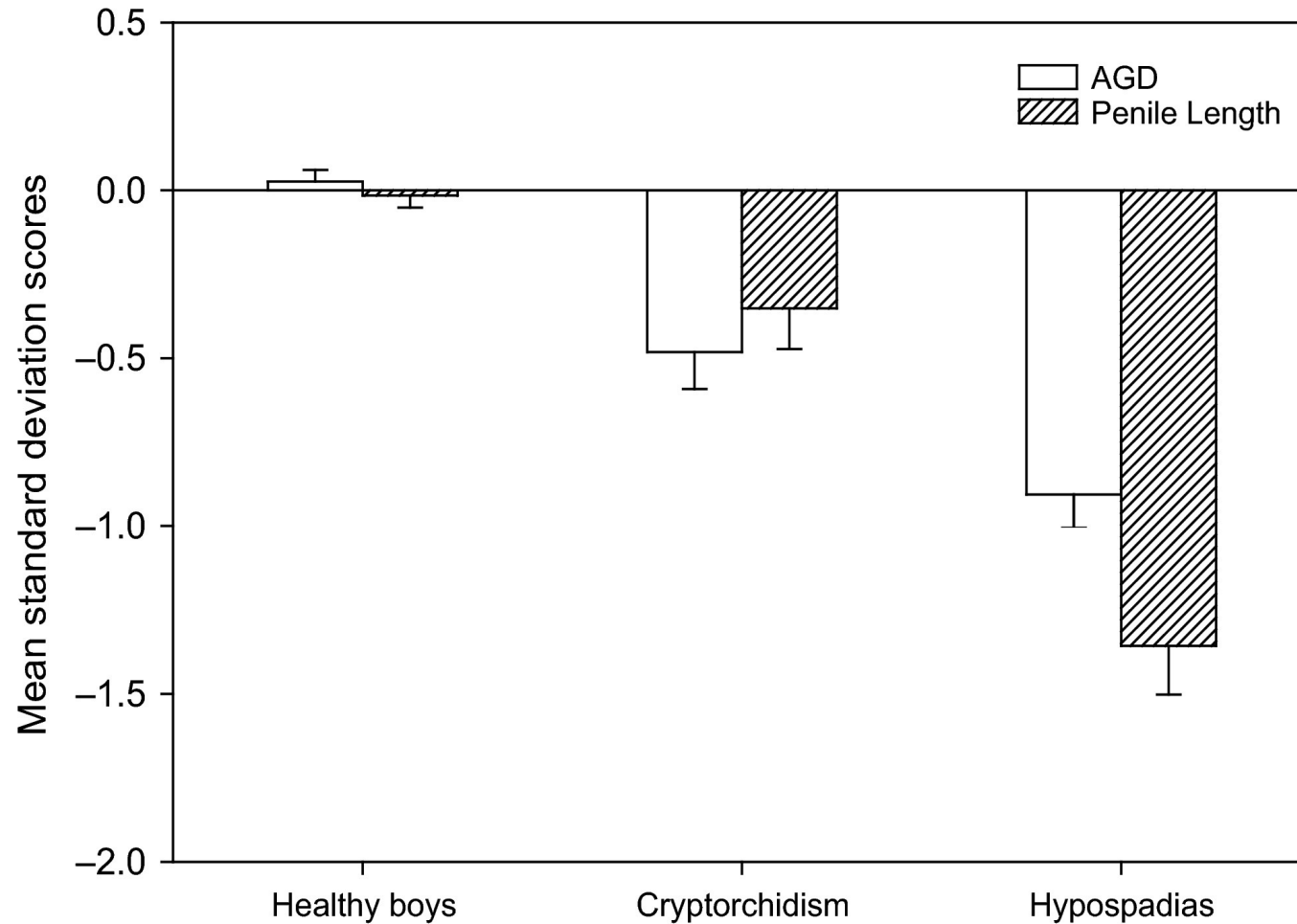
SCAHT, Swiss Center for Applied Human Toxicology  **scaht**

anogenital distance as an anthropomorphic marker of exposure to androgens versus estrogens



association of shortened anogenital distance (AGD) with clinical manifestations at young and adult age

- testicular dysgenesis syndrome, Skakkebaek *et al.* (2022) *Nat. Rev. Endocrinol.* 18: 139-157 -

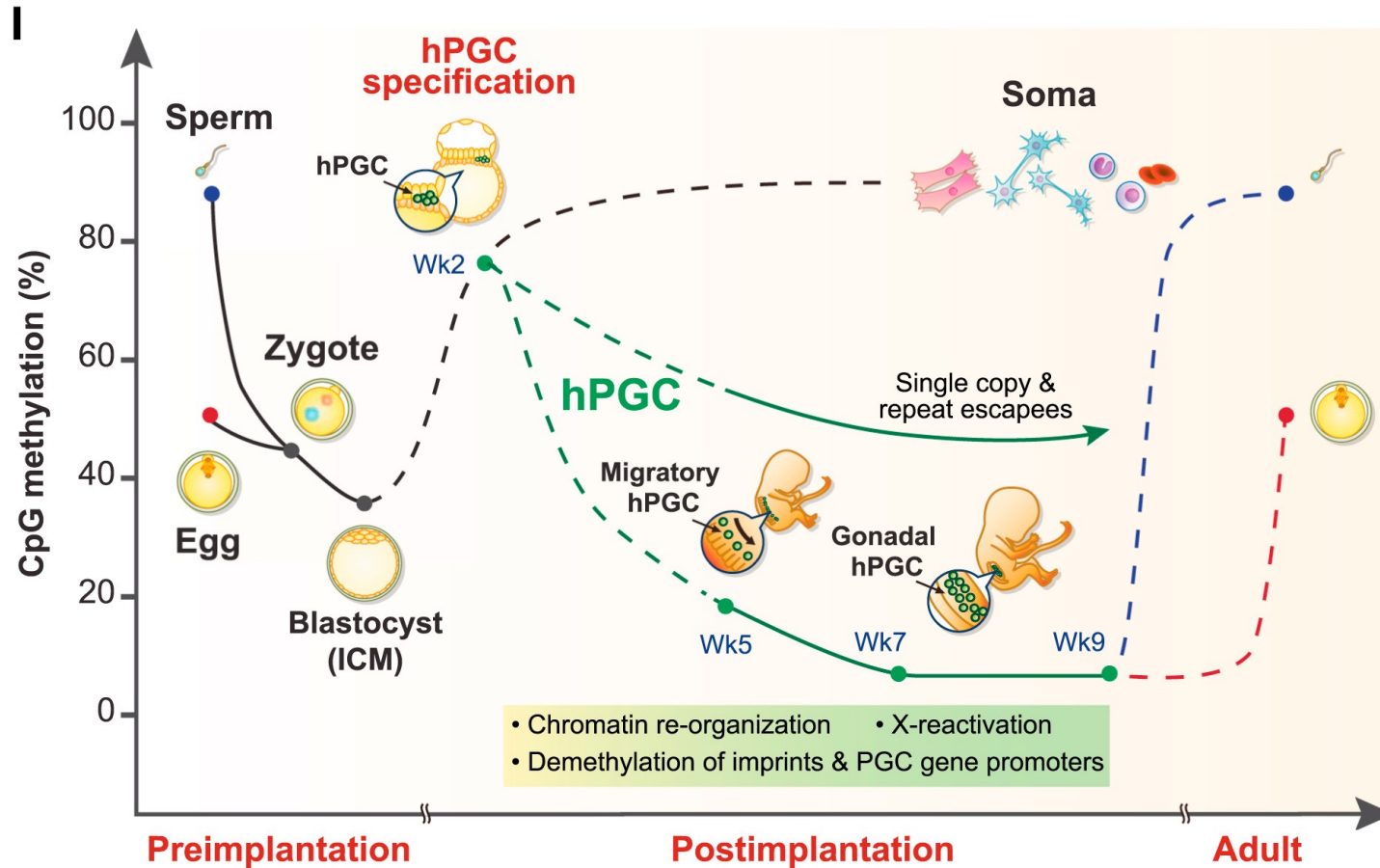


anogenital distance and endocrine disruptors

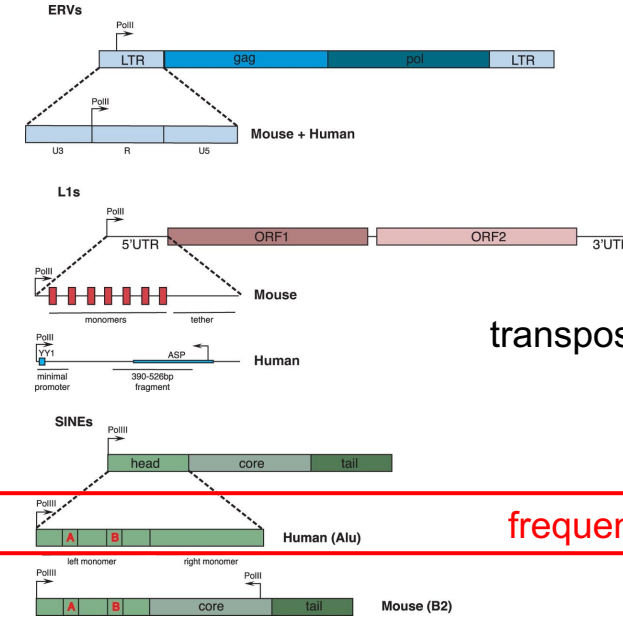
chemical	outcome	case load	ref.
Bisphenol A	low sperm count & motility	218	Li <i>et al.</i> (2011)
	low sperm count & motility	190	Meeker <i>et al.</i> (2010)
	reduced semen quality	375	Mendiola <i>et al.</i> (2010)
	shortened anogenital distance	153	Miao <i>et al.</i> (2011)
Phthalates	reduced sperm motility	180	Pant <i>et al.</i> (2011)
	low sperm count & motility	150	Liu <i>et al.</i> (2012)
	low sperm count & motility	463	Hauser <i>et al.</i> (2006)
	low sperm count, motility & morphology	168	Duty <i>et al.</i> (2003)
	shortened anogenital distance, reduced penile width	371	Swan <i>et al.</i> (2015)

estrogenic signaling, including Alu sequences

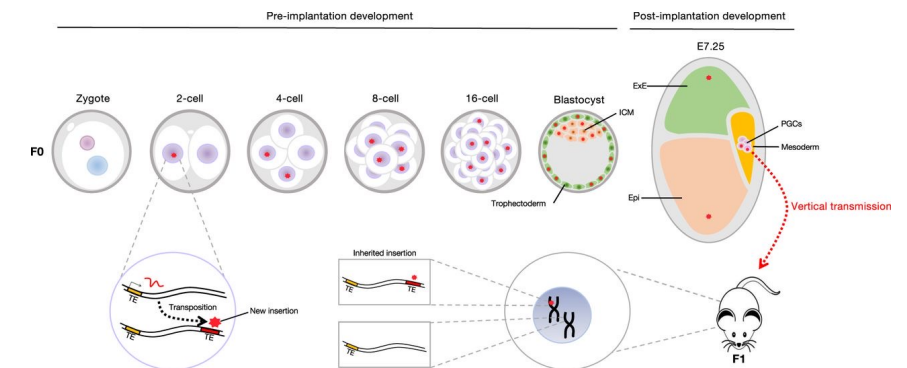
During postimplantation development the genome of hPGC (human primordial germ cells) undergoes extensive demethylation of imprints.



Tang *et al.* (2015) Cell 161: 1453-1467



transposable elements (TEs)



Hermant & Torres-Padilla (2021) Genes Dev. 35:22-39

hypothesis

We hypothesized that during antenatal development primordial germ cells in infertile men with shortened anogenital distance (AGD) may have been exposed to endocrine disruptors.

During primordial germ cell development global DNA demethylation takes place potentially allowing transposable elements containing estrogen-response elements (ERE) to become integrated in the genome of proliferating germ cells of post-implantation embryos.

Changes in DNA methylation in the genomes of spermatozoa from infertile men with shortened AGD may be associated with differentially methylated transposable elements (e.g. Alu) containing estrogen-response elements (ERE).

Norris *et al.* (1995) J. Biol. Chem. 270: 22777-22782

Babich *et al.* (1999) Gene 239: 341-349

Oei *et al.* (2004) Genomics 83: 873-882

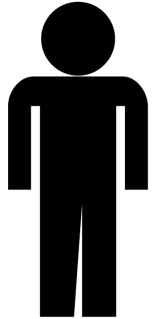
aim of the study



Primordial germ cells

- **Epigenetic reprogramming**
- **Sexual differentiation**

-> *DNA methylation* Reduced representation bisulfite sequencing
RRBS
-> *anogenital distance (AGD)*



Spermatozoa

- **Histone-protamine remodeling**
- **Apoptosis**

-> *FACS + CMA3 dye*
-> *FACS + YOPRO1 dye*



To study ***DNA methylation*** interacting with ***apoptosis & histone to protamine remodeling*** in male partners of ***infertile*** couples selected for ***short AGD***

The study protocol was approved by the local ethics committee: EKNZ 2017-01407.
The study was funded by SCAHT:

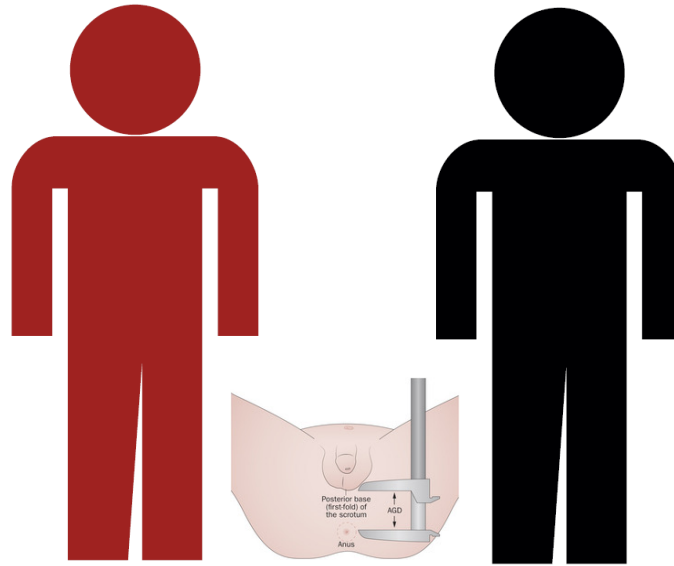


study cohort

infertile patients

couples that failed to
conceive after one year

short AGD < 40 mm



fertile donors

couples that achieved
pregnancy within three
months

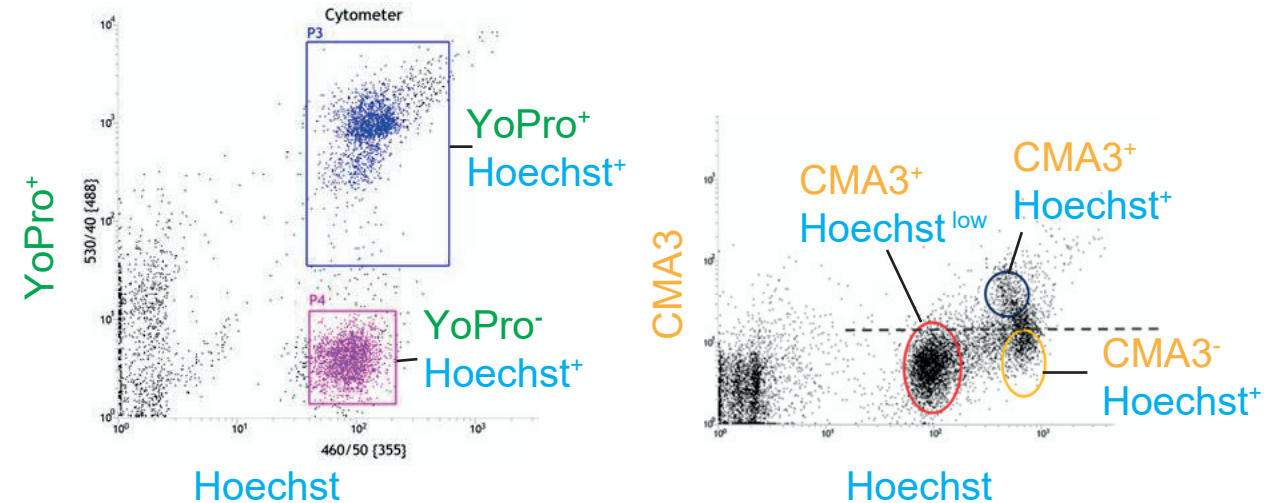
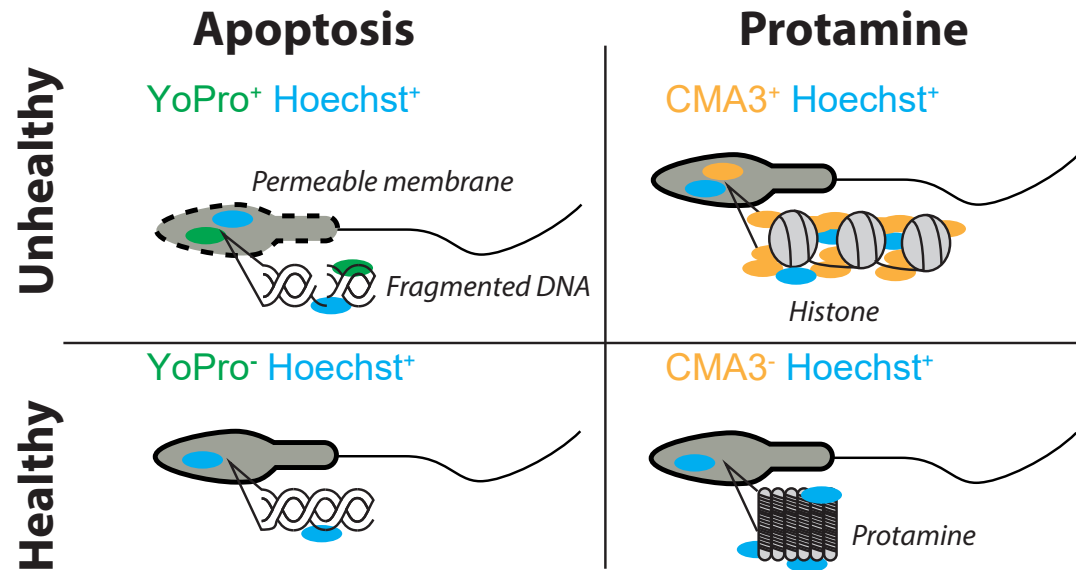
normal AGD > 40 mm

testicular volume, gonadal ultrasound, body mass index (BMI), blood pressure, LH, FSH, T, conventional semen analysis, TUNEL

characteristics of the participants recruited to the study

	infertile patients (n=11)				healthy semen donors (n=10)				sign.
parameter	median	mean	SD	range	median	mean	SD	range	p-value
age (y.)	42.0	41.5	4.9	35-50	33.0	34.9	5.8	27-44	0.006
BMI (kg/m ²)	24.9	24.8	3.3	21.7-31.4	23.3	23.5	2.9	20.0-29.3	0.123
RR systolic (mm Hg)	128.0	129.5	15.6	109-155	124	127.4	14.8	111-157	0.429
RR diastolic (mm Hg)	74.0	75.1	9.6	63-93	75.5	73.5	10.2	55-91	0.500
AGD (mm)	35.0	35.0	7.4	25-44	69.0	70.2	8.2	58-87	<0.0001
testicular volume (ml)	42.0	33.9	11.8	22-55	69.0	50.0	51.0	40-70	0.004
LH (IU/l)	4.5	4.7	1.4	3.0-7.7	5.1	4.8	1.3	2.5-6.5	0.363
FSH (U/l)	5.2	4.9	1.8	1.8-8.0	2.7	3.7	2.1	1.7-8.1	0.066
testosterone (nmol/l)	14.9	18.1	9.2	7.0-33.6	21.2	20.5	5.4	12.6-30.6	0.138
sperm concentration (mill./ml)	43.7	69.4	48.9	19.3-158	57.1	71.3	55.7	18.1-211	0.484
progressive motility (%)	44.0	42.2	20.2	14-80	44.5	47.0	14.0	28-72	0.298
normal morphology (%)	13.6	12.5	5.8	1.8-21.6	10.5	11.3	4.3	5.9-18	0.323
DNA-fragmentation (%)	32.5	29.5	8.6	22.1-51.5					

sorting of washed spermatozoa of healthy donors and infertile patients with FACS into 4 populations



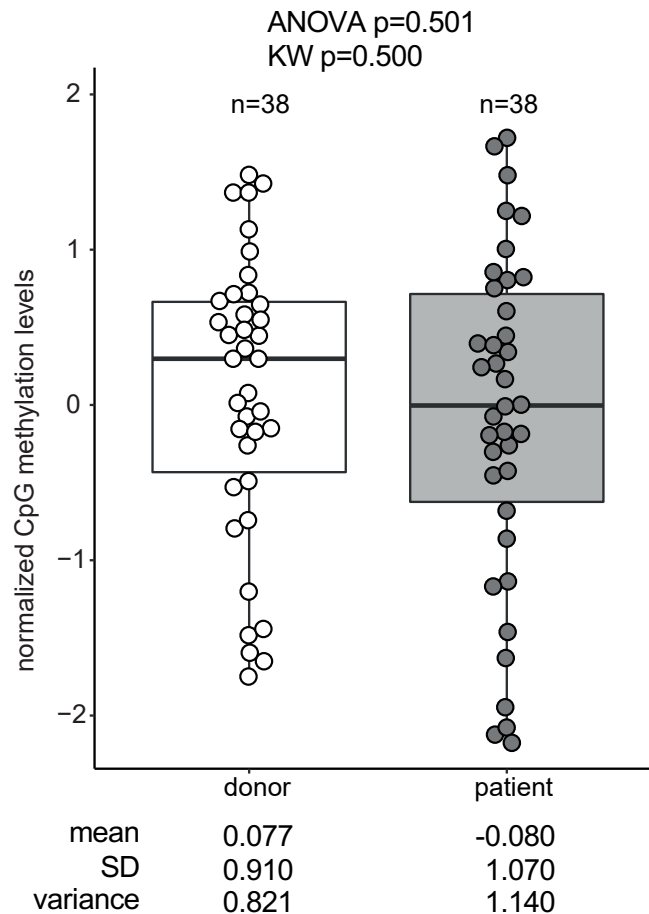
representative examples of sorting with FACS

then:

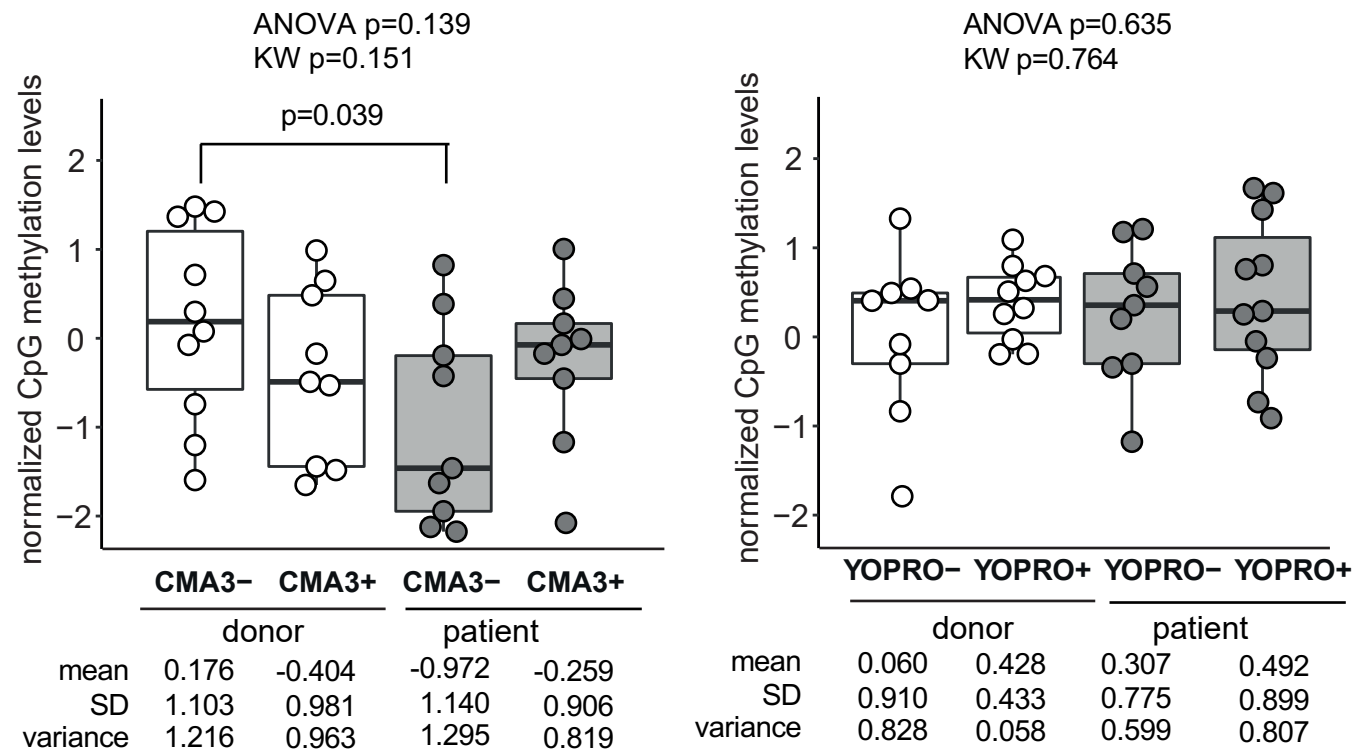
- DNA extraction of the four FACS-sorted populations.
- Reduced representation bisulfite sequencing (RRBS) for whole genome cytosine methylation determination.
- Analysis of RRBS data in the high performance computing cluster of the University of Geneva.
- Identification of estrogen-responsive elements (ERE): Alu sequences with at least 1 ESR1-binding site with threshold for genome-wide significance set at >7.6 .

results

global characterization of methylation status of the washed and sorted sperm populations (1)



global characterization of methylation status of the washed and sorted sperm populations (2)



CMA3-uptake into sperm of infertile men vs. healthy donors

	ERE CMA3- vs. CMA3+		no ERE CMA3- vs. CMA3+		ERE patients vs. donors		no ERE patients vs. donors	
hypo Alu	-1.90	2.27	0.87	-1.04	0.04	-0.20	-0.02	0.11
hypo	0.02	-0.05	0.00	0.01	0.08	-0.08	-0.01	0.04
hyper	1.97	-0.67	-0.21	0.07	0.03	-0.05	0.00	0.00
hyper Alu	-2.02	0.55	0.89	-0.24	-3.87	2.13	1.77	-0.97

The number of hypomethylated estrogenic Alu sequences in the condensed (CMA3-positive) sperm fractions was significantly higher than expected ($p=0.0137$).

The number of hypermethylated estrogenic Alu sequences was significantly lower in the sperm fractions of infertile patients ($p=0.0003$).

YOPRO1-uptake into sperm of infertile men vs. healthy donors

	ERE		no ERE		ERE		no ERE	
	YOPRO- vs. YOPRO+		YOPRO- vs. YOPRO+		patients vs. donors		patients vs. donors	
hypo Alu	-3.71	5.40	2.03	-2.96	0.03	-0.03	-0.01	0.01
hypo	2.63	-4.01	-0.25	0.38	0.25	-0.21	-0.04	0.03
hyper	-0.17	0.03	0.02	-0.04	-0.16	0.11	0.01	-0.01
hyper Alu	-0.03	0.38	-0.01	-0.04	4.11	-1.52	-1.79	0.66

The number of hypomethylated estrogenic Alu sequences in the apoptotic (YOPRO1-positive) sperm fractions was significantly higher than expected ($p=0.0002$).

The number of hypermethylated estrogenic Alu sequences was significantly higher in the sperm fractions of infertile patients than expected ($p=0.004$).

conclusion and limitations

- Cohorts of spermatozoa of infertile men with short AGD, as a marker of antenatal exposure to anti-androgenic or estrogenic endocrine disruptors, carry differentially methylated regulatory segments of genes that may be involved in sperm function, such as chromatin density and apoptosis.

conclusion and limitations

- Cohorts of spermatozoa of infertile men with short AGD, as a marker of antenatal exposure to anti-androgenic or estrogenic endocrine disruptors, carry differentially methylated regulatory segments of genes that may be involved in sperm function, such as chromatin density and apoptosis.
- Limitations of the study are: ... the inclusion of few participants (n=21),
- ... the unproven association of short AGD and exposure to endocrine disruptors,
- ... the limited diagnostic accuracy of RRBS (70-80 %),
- ... the indirect association of CMA3 with chromatin density and of YO-PRO1 with apoptosis,
- ... the focus on ERE only in the regulatory sequences of the genome.