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PERIPUBERTAL SERUM DIOXINS AND SUBSEQUENT ADULT SEMEN QUALITY AND SPERM METHYLOME IN THE PROSPECTIVE RUSSIAN CHILDREN’S STUDY

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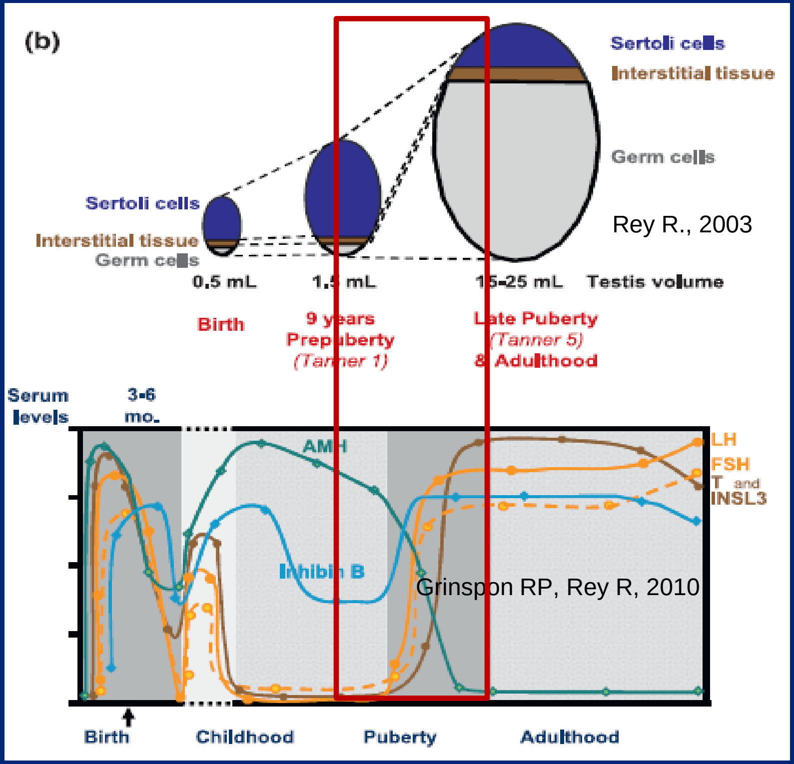
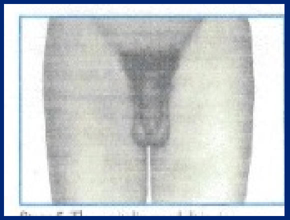
Disclosure

- I have nothing to disclosure

Peripuberty – vulnerable period

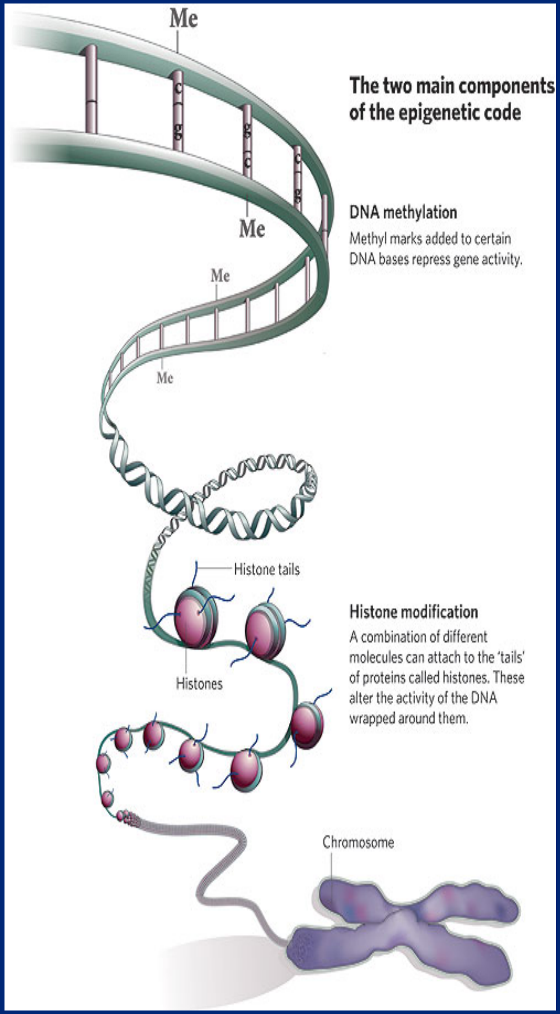
- Peripubertal period:
  - 1-2 years prior pubertal onset
  - Activation of HPG axis
  - Proliferation of Sertoli cells and spermatogonia
- Spermatogenesis:
  - Hormone dependent
  - Spermarche occurs 1-2 yrs after pubertal onset (13-15 years)
  - Duration – appox 60-74 days
- Puberty – period of dramatic changes
  - ↑ expression of genes encoded hormones and its receptors
- Vulnerable to endocrine disrupting chemicals (EDCs)





Epigenetics

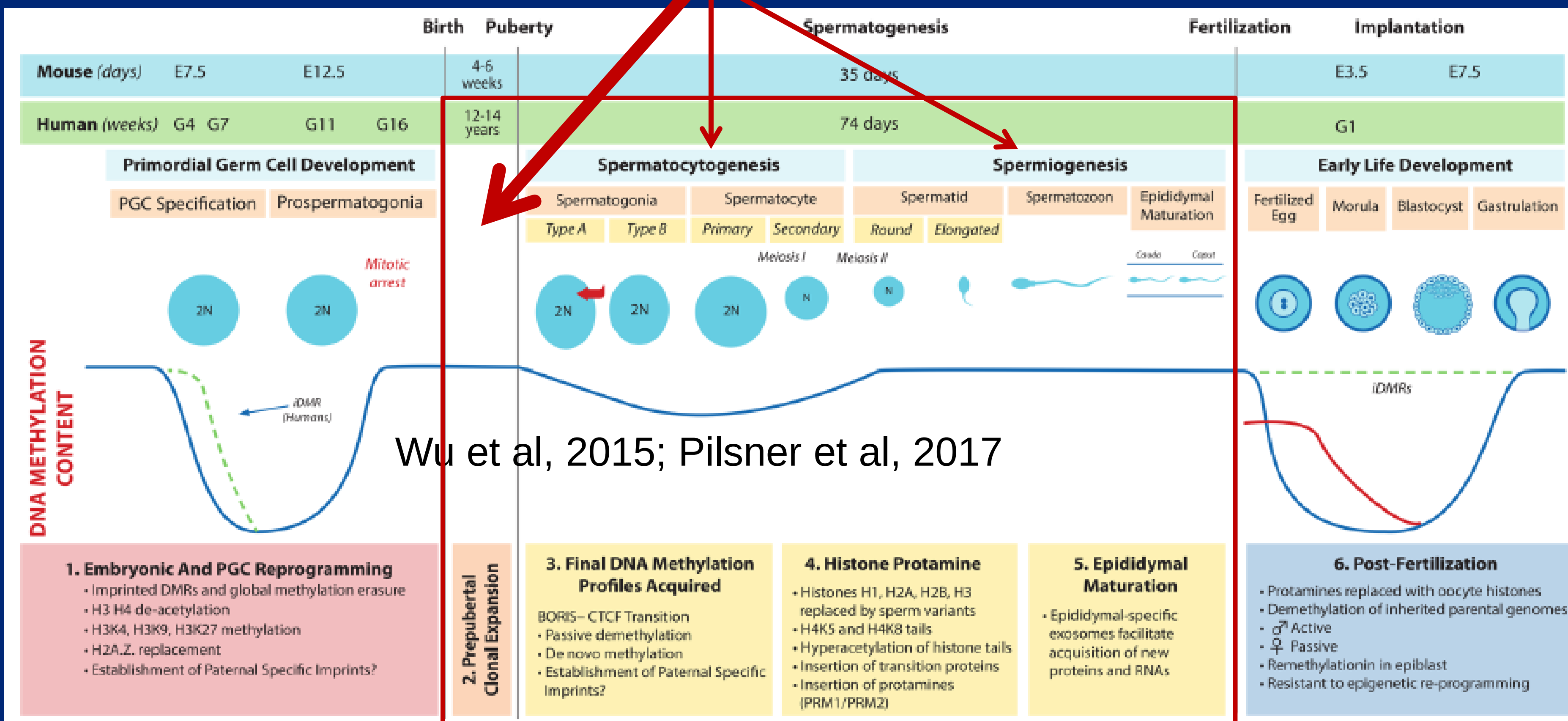
- Definition
  - Changes in gene expression without change in DNA sequence
- Epigenetic marks
  - **DNA methylation**
    - Addition of methyl groups to the cytosine in CpG dinucleotides
  - Histone modifications
  - non-coding RNAs
- Impact chromatin packaging and thus accessibility of transcription factors to promoters and enhancers
- Cell specificity
- A mediator of the genome to bring upon phenotype



Qui, J; *Nature* 441; 2006

Sperm epigenome

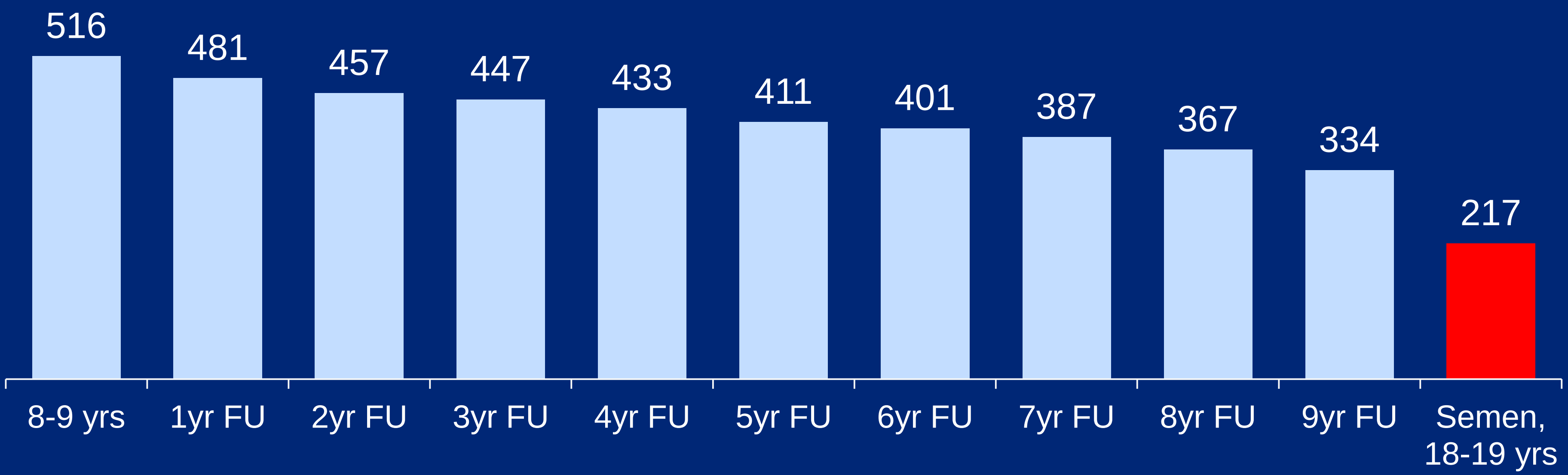
- Specific windows of sperm development and epigenome
  - Early development *(in utero)*
  - **Peripubertal period (7-16 years) → spermatogenesis**
- Sensitive to endocrine disrupting chemicals (800+ chem)



Wu et al, 2015; Pilsner et al, 2017

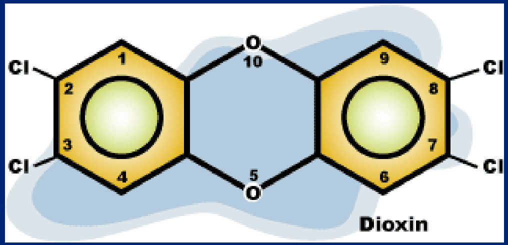
Longitudinal Male Cohort Study in Chapaevsk, Russia

- Longitudinal male cohort study with **annual** assessment of growth, puberty and semen quality
- Conducted in Chapaevsk, small industrial city, Samara region, central Russia, population 72,000
- Russian Children's Study started at 2003
- 8-9 yrs Chapaevsk boys born in 1994, 96, 97 – **90% of all eligible**
- 516 subjects; 4700+ exams; 20000+ sample aliquots, semen – 42%



Why focus on dioxins?

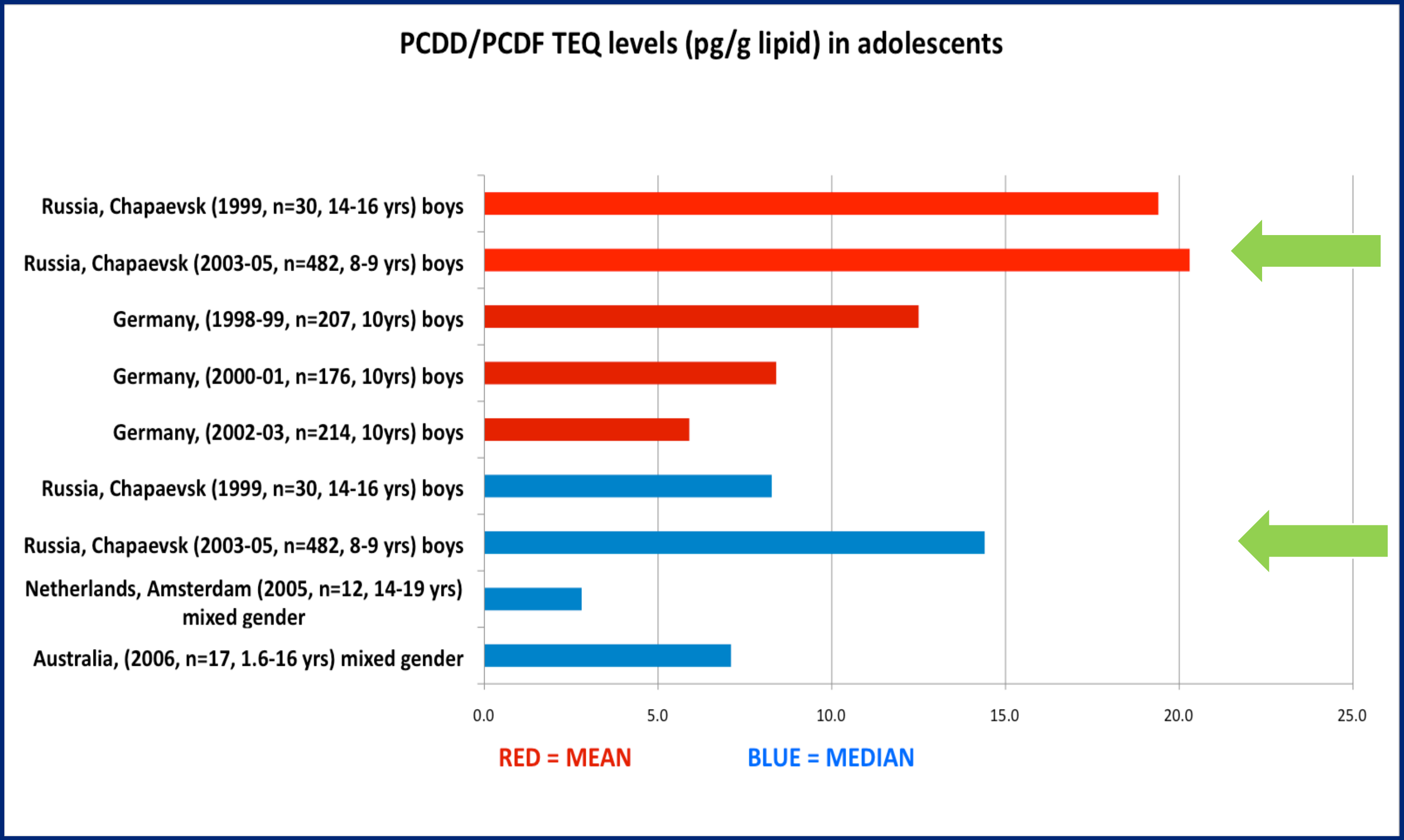
- Dioxins - Persistent Organic Pollutant (POPs)
  - Still distributed widely in the environment with long-range transport
    - **Everyone has background exposure level**
  - Toxic and lipophilic , TCDD is a most toxic congener
  - Unwanted by-products of chemical industry, combustion (incineration)
  - Environmentally and biologically persistent
    - Half-life in children is 7-10 years
  - Bioaccumulation/biomagnification



- Dioxins - Endocrine Disrupting Chemicals
  - Interfere in:
    - Secretion, direct action on hormone receptor and function
  - Low-dose effect, pg/g – doses comparable with estradiol and free testosterone level in male (pg/ml)
  - Can act through aryl hydrocarbon receptor (AhR) –"dioxin" receptor

Peripubertal Dioxins Level, RCS

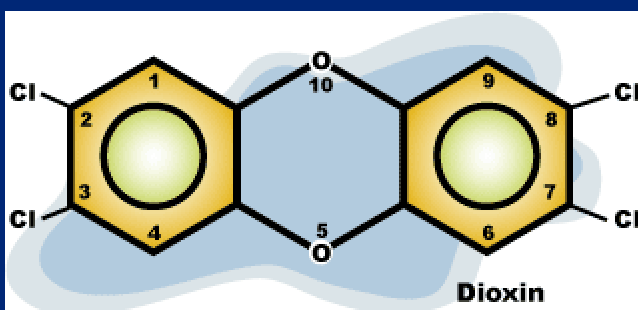
- Boy's serum at 8-9 yrs analyzed for
  - TCDD -6/10 Dioxins/Furans - 41 PCB congeners
- Wide range of dioxins level in cohort



Dioxins and pubertal outcomes in RCS

- Later pubertal onset (Korrick et al. 2011, Burns et al, 2016)
- Later sexual maturity (Burns et al. 2016)
- Later growth and development (Burns et al. 2011)

Dioxins and semen quality



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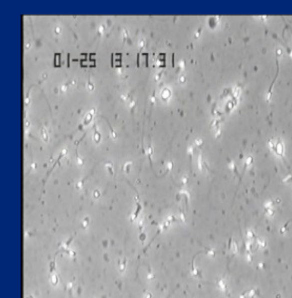
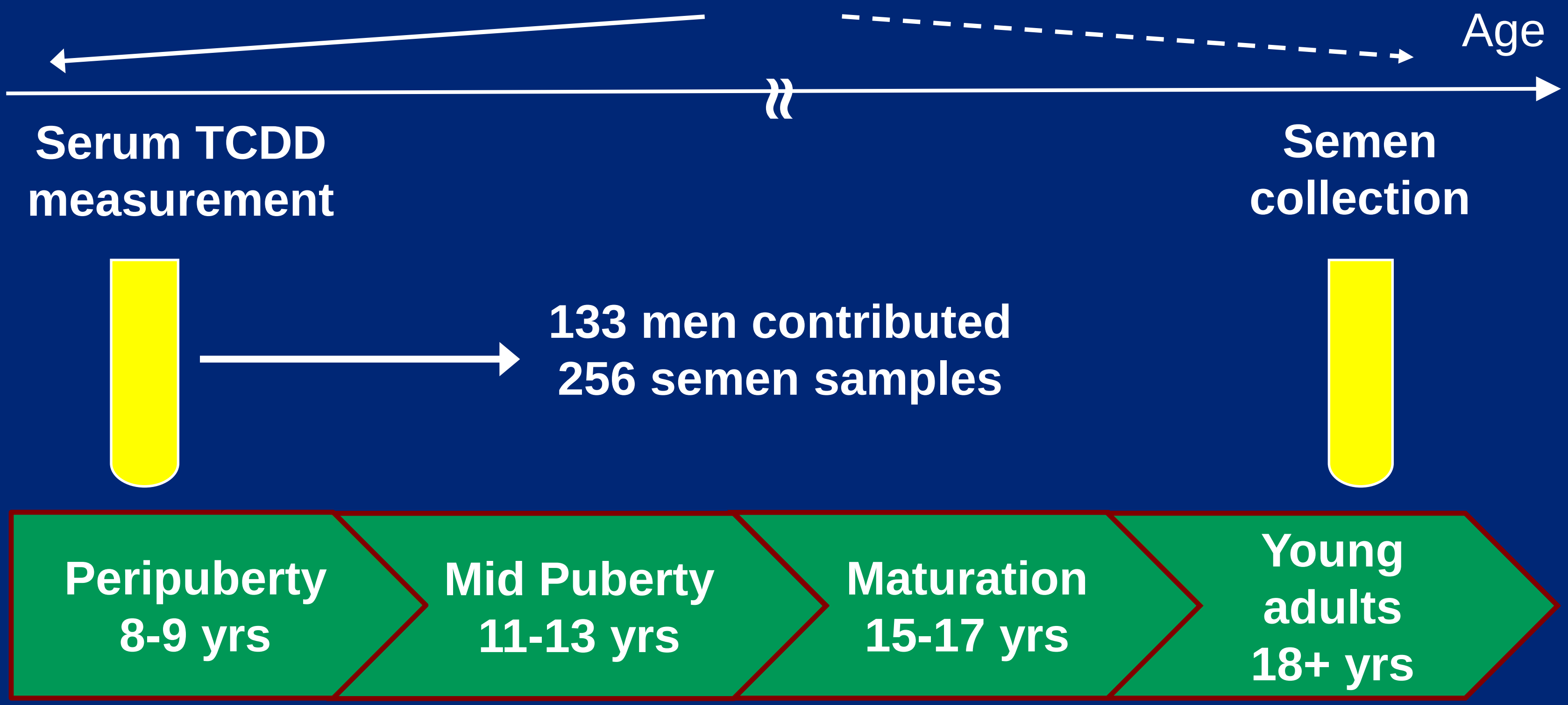
Seveso study and…..



Objective

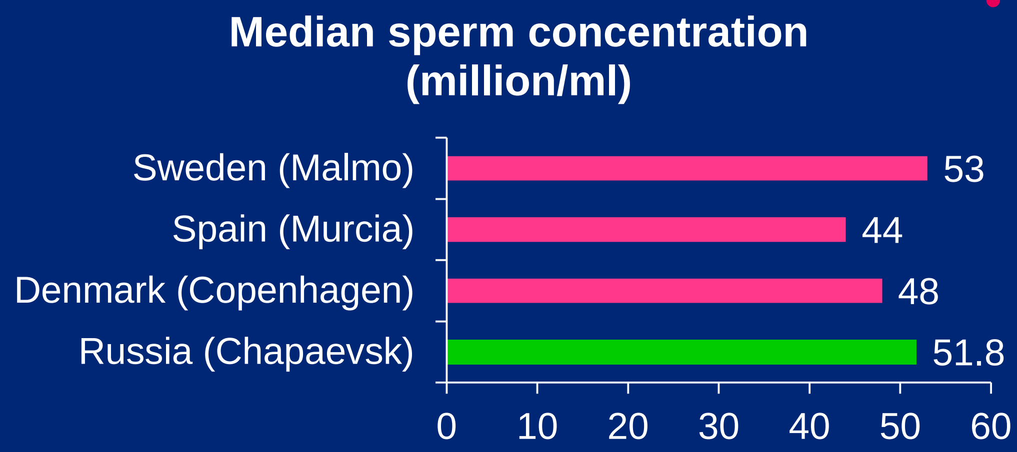
- To investigate the relationship between peripubertal serum dioxin (TCDD, most toxic) levels and semen quality

Yearly physical examinations and sample collection



Semen parameters among young adults

Parameter	Russia (Chapaevsk)	Denmark (Copenhagen)[1]	Spain (Murcia)[2]	Sweden (Malmo)[3]
Period of sampling	2012-2015	2006-2010	2011-2012	2008-2010
Number of subject	133	4867	215	112
Age (years)	18.3	19	20.4	18.3
Semen volume (ml)	2.4	3.3	3	2.6
Sperm concentration (million/ml)	51.8	48	44	53
Total sperm count (million)	127	151	121	140
Motility (%)	64	68	57.2	-



- In general, young Russian men have slightly better semen parameters than young Spanish men[2] and comparable with Danish[1] and Swedish[3] men

[1] Jorgensen et al, 2012
[2] Mendiola et al, 2013
[3] *Axelsson et al, 2015*

Peripubertal Dioxins and Semen Quality

Seveso study, n=71[1]:

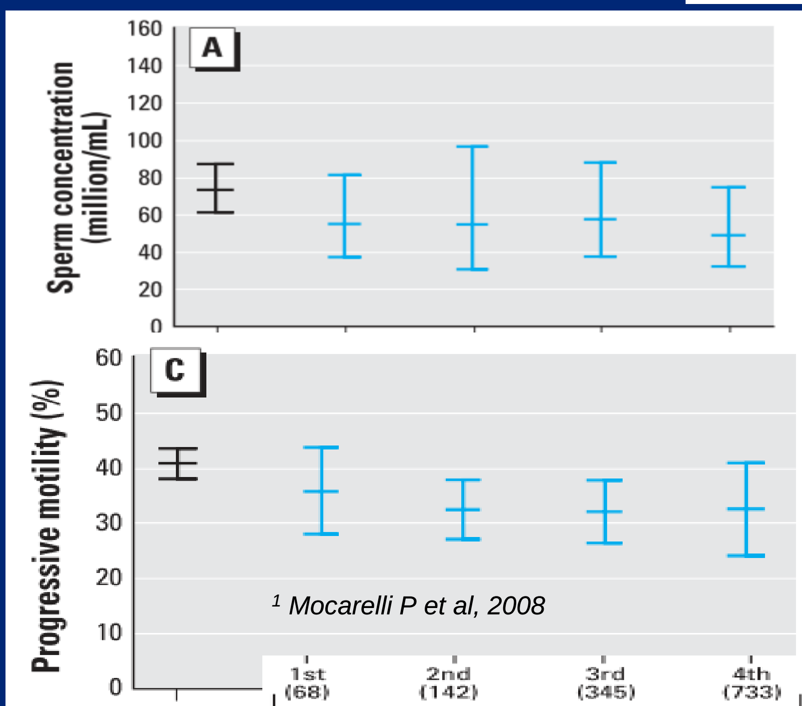
- mean age - 6.2 years
- acute exposure
- median serum TCDD - ***210 pg TEQ/g lipid***

Different exposure scenario

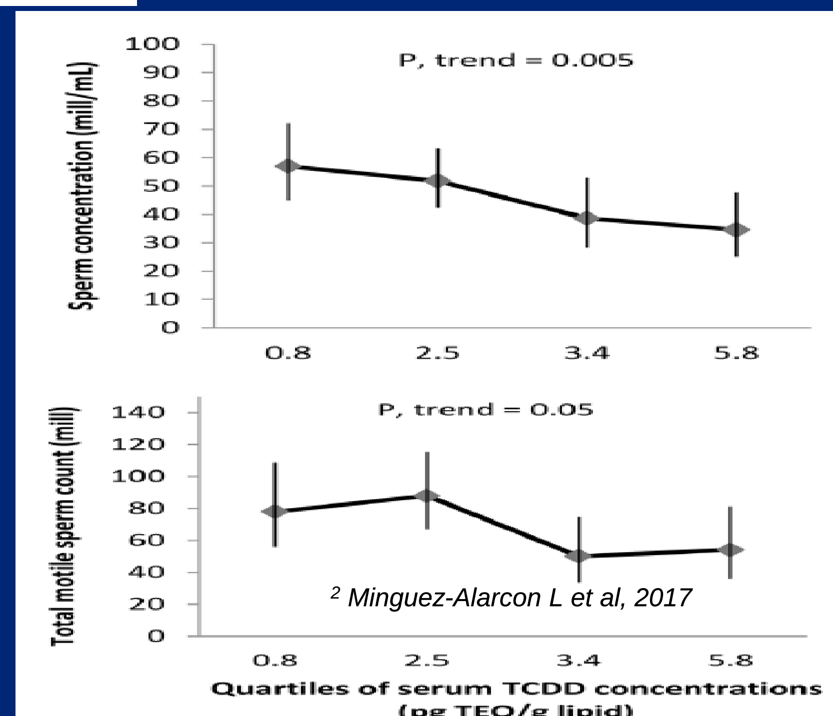
70 fold lower

RCS study, n=133[2]:

- mean age - 8.4 years
- background exposure
- median serum TCDD - ***2.9 pg TEQ/g lipid***

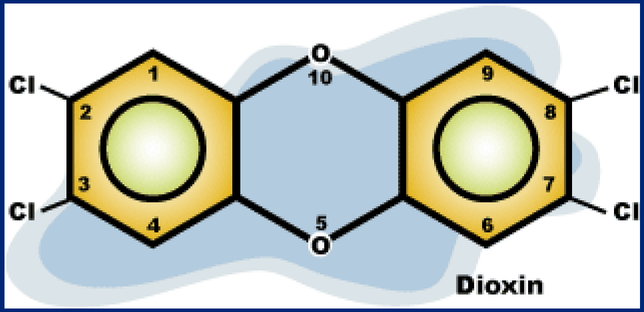


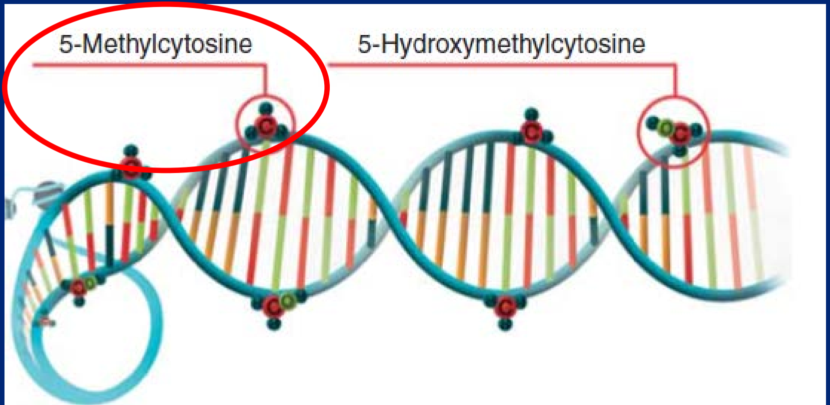
[1] Mocarelli P et al, 2008



[2] Minguez-Alarcon L et al, 2017

Dioxins and sperm methylome

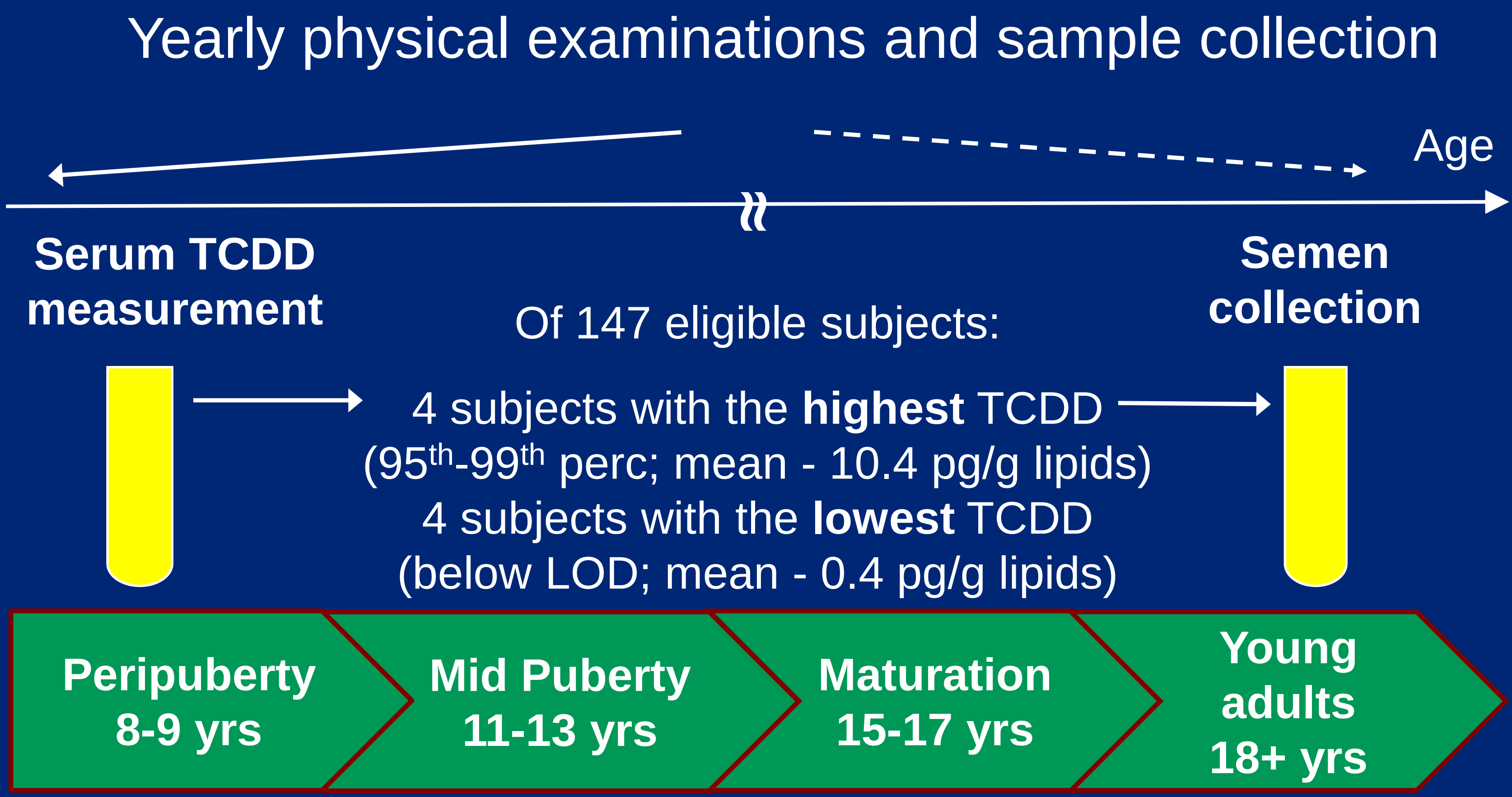




Jenkins and Carrell. Reproduction (2012) 143

Objective for epigenetics part

- To examine the relationship between peripubertal dioxin (TCDD, most toxic) levels and genome-wide profiles of DNA methylation in sperm collected in young adulthood



Methods

- Thawed 2nd sperm samples without cryoprotectant

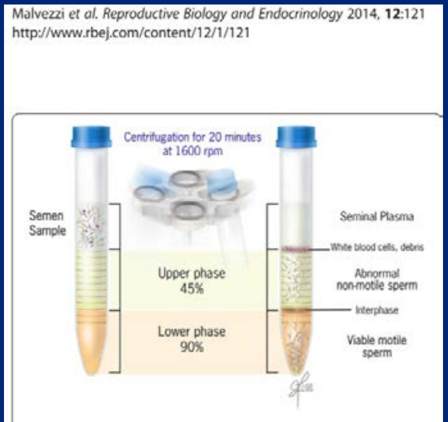
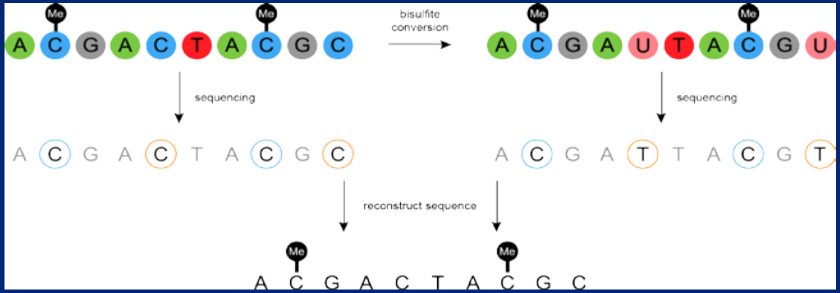


Figure 2 Schematics of sperm preparation by density gradient separation.

- Sperm gradient separation
  - “Isolate” (Irvine Scientific) 90 and 50% density
- DNA isolation from sperms (Wu et al. 2015) with modifications
- **Whole Genome Bisulfite Sequencing**
- Bisulfite conversion - EZ DNA Methyl-Lightning Kit (Zymo Research)
- Library preparations (100-150 ng) - TruSeq DNA Methyl Kit, Illumina
- HiSeq 2500 (Illumina)
  - 216 mill reads per sample
- All CpGs - average coverage ~ **3x**



- **Only CpGs with ≥10 coverage included** (ENCODE, 2011)

Pilsner et al, 2018

Lowest vs Highest TCDD groups

Characteristic [mean (range)]	Lowest TCDD, n=4	Highest TCDD, n=4
Age (years)	18.8 (18.1-19.3)	18.7 (18.1-19.1)
BMI (kg/m^2)	22.1 (19.4-23.6)	21.0 (18.7-23.6)
Smoking status	0 (0)	0 (0)
Total daily dietary intake, kcal	2828 (2059–3539)	3353 (2716–4011)
Mean testicular volume (ml)	24.4 (20-28.8)	23.1 (17.5-28.8)
Semen volume (ml)	3.9 (0.8-9.2)	5.0 (1.5-7.9)
Motility, a+b+c (%)	59.3 (48-66)	65.3 (64-68)
Sperm concentration (ml/mill)	89.8 (53-115.6)	49.3 (13.9-82.3)
Total motile sperms (mill)	170.1 (34.2-251.8)	105.0 (68.5-144.3)
Specific parameters of WGBS		
Bisulfite conversion efficiency (%)	98.8	98.8
Total unique reads	116 750 000	98 750 000
Alignment, %	60.5	67.5

Pilsner et al, 2018

Lowest (LE) vs Highest (HE) TCDD groups

- The mean methylation across all CpG sites lower in the HE group compared to LE (49 ± 5% and 62 ± 5% ; $p = 0.01$)

- Criteria for Differentially Methylated CpGs:
  - **≥ 10x coverage depth; ≥ 10% methylation change; q value <0.05**

↓

- 666 individual CpGs differentially methylated

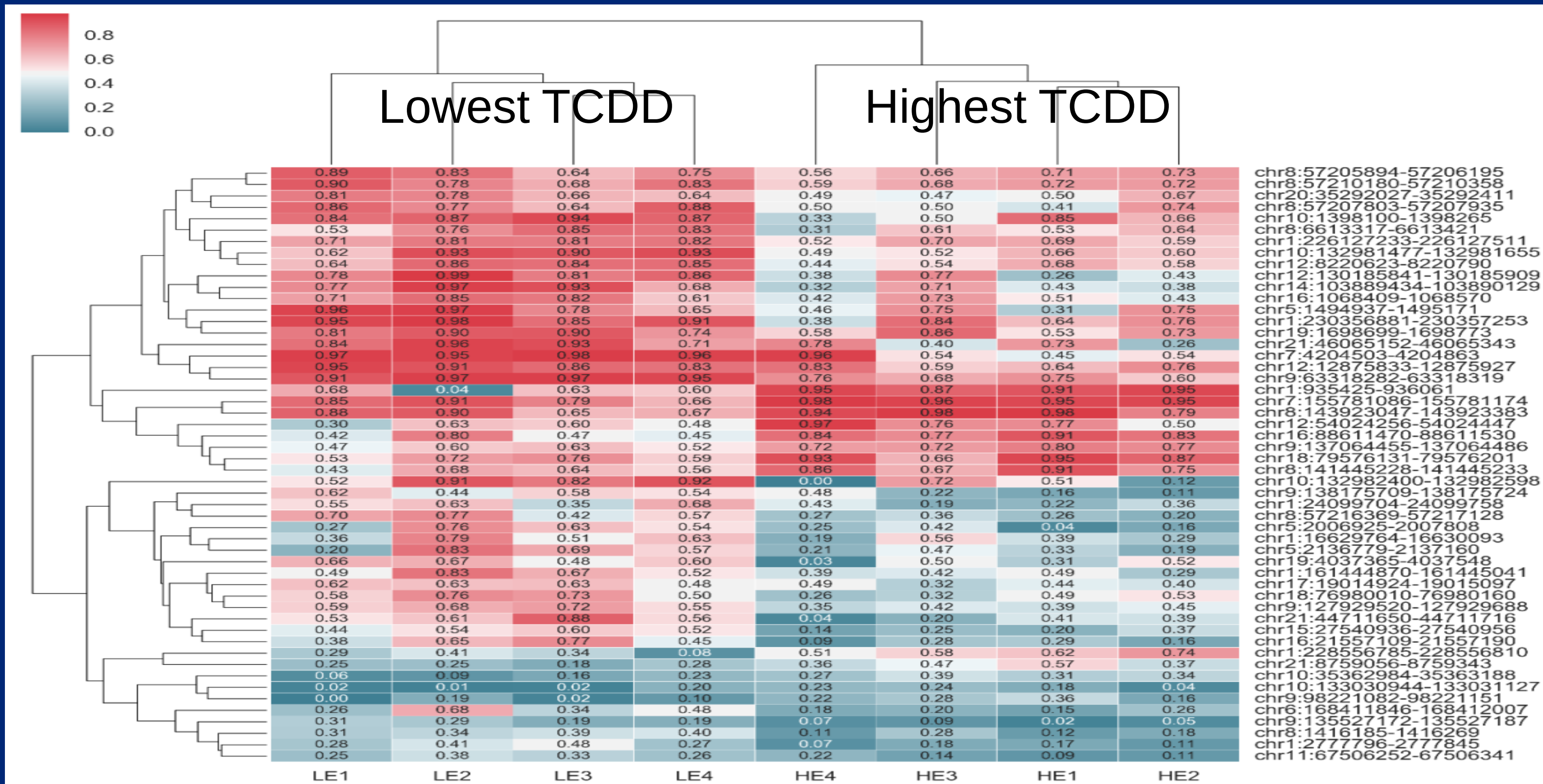
- Restriction to regions with ≥ 3 CpGs per cluster:

↓

52 differentially methylated regions (DMRs)

Pilsner et al, 2018

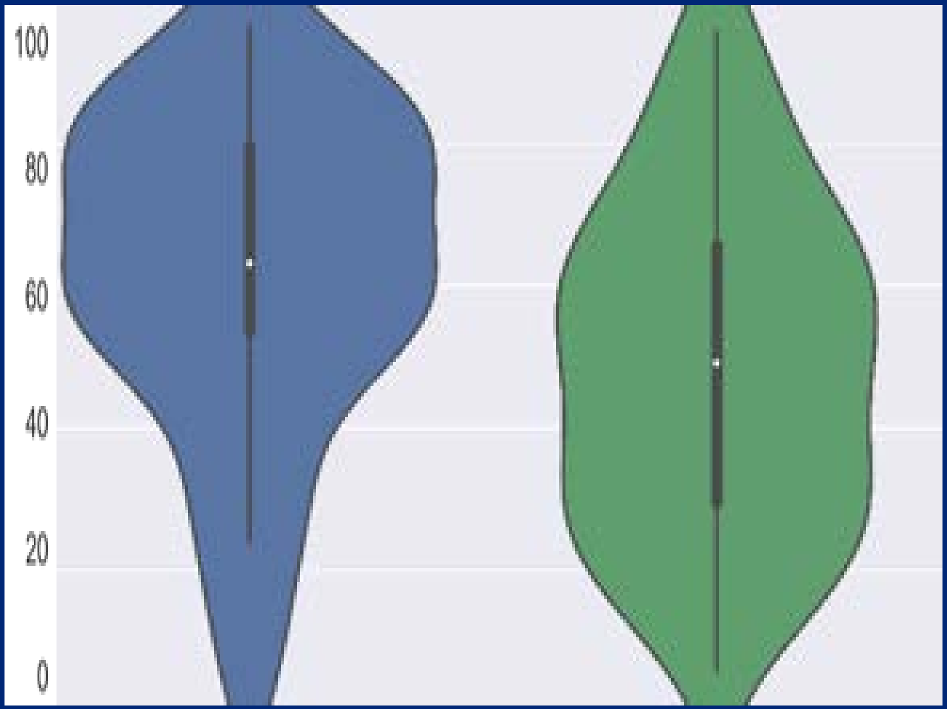
WGBS – 52 DMRs in two groups

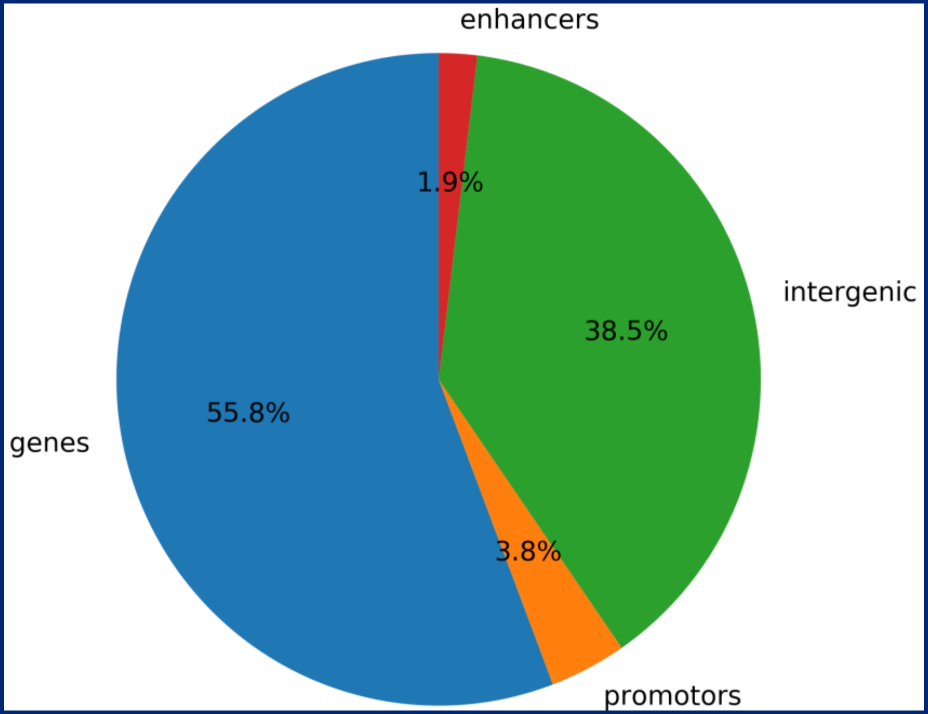


- Clustering and heatmap of the 52 significant DMRs
- High percent methylation is shown in red while low percent methylation is in blue
- 75% of the DMRs were hypomethylated in HE compared to LE

Pilsner et al, 2018

WGBS – 52 DMRs in two groups





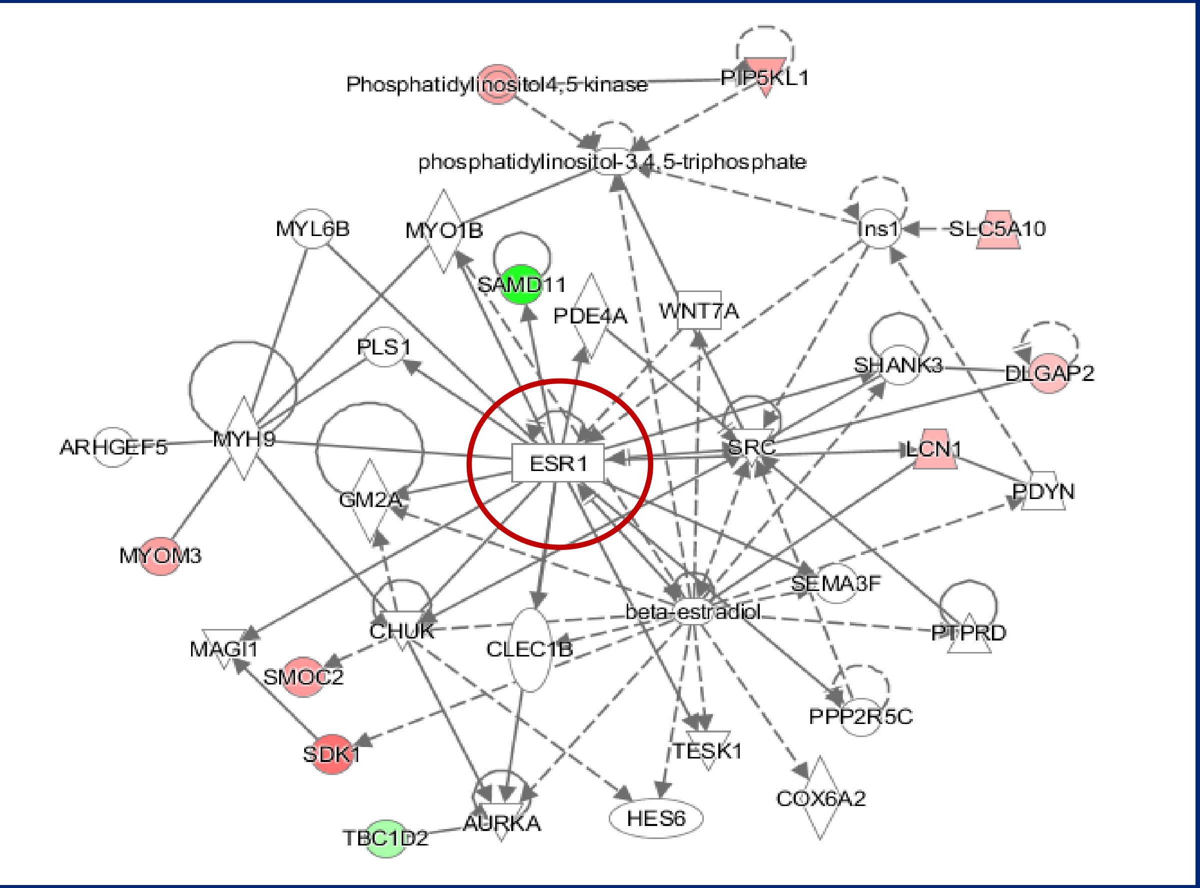
Lowest Highest

- Across 52 DMRs:

- The mean methylation lower in the HE group compared to LE - 49.1 and 63.2
- The majority of sperm DMRs were located in gene bodies (56%) and intergenic regions (38%)

Pilsner et al, 2018

ESR1 – key regulator



- Integrity Pathway Analysis (IPA; Qiagen)– functional enrichment analysis
- Top scoring networks, “Cellular Assembly and Organization, Cellular Function and Maintenance, Carbohydrate Metabolism”
  identified **estrogen receptor alpha (ESR1) as its central regulator**

Pilsner et al, 2018

Strengths and Limitations

- Limitations:
  - Small number of subjects (other factors can affect)
  - Relatively low depth of coverage (average ~ 3x) across the genome and restriction of analyses to regions with ≥ 10x depth of coverage - 13% genome-wide coverage
- Strengths:
  - Longitudinal design of well-established cohort
  - Selected subjects with the lowest and highest peripubertal serum TCDD concentrations in population-based cohort
- Future directions:

Pilsner et al, 2018

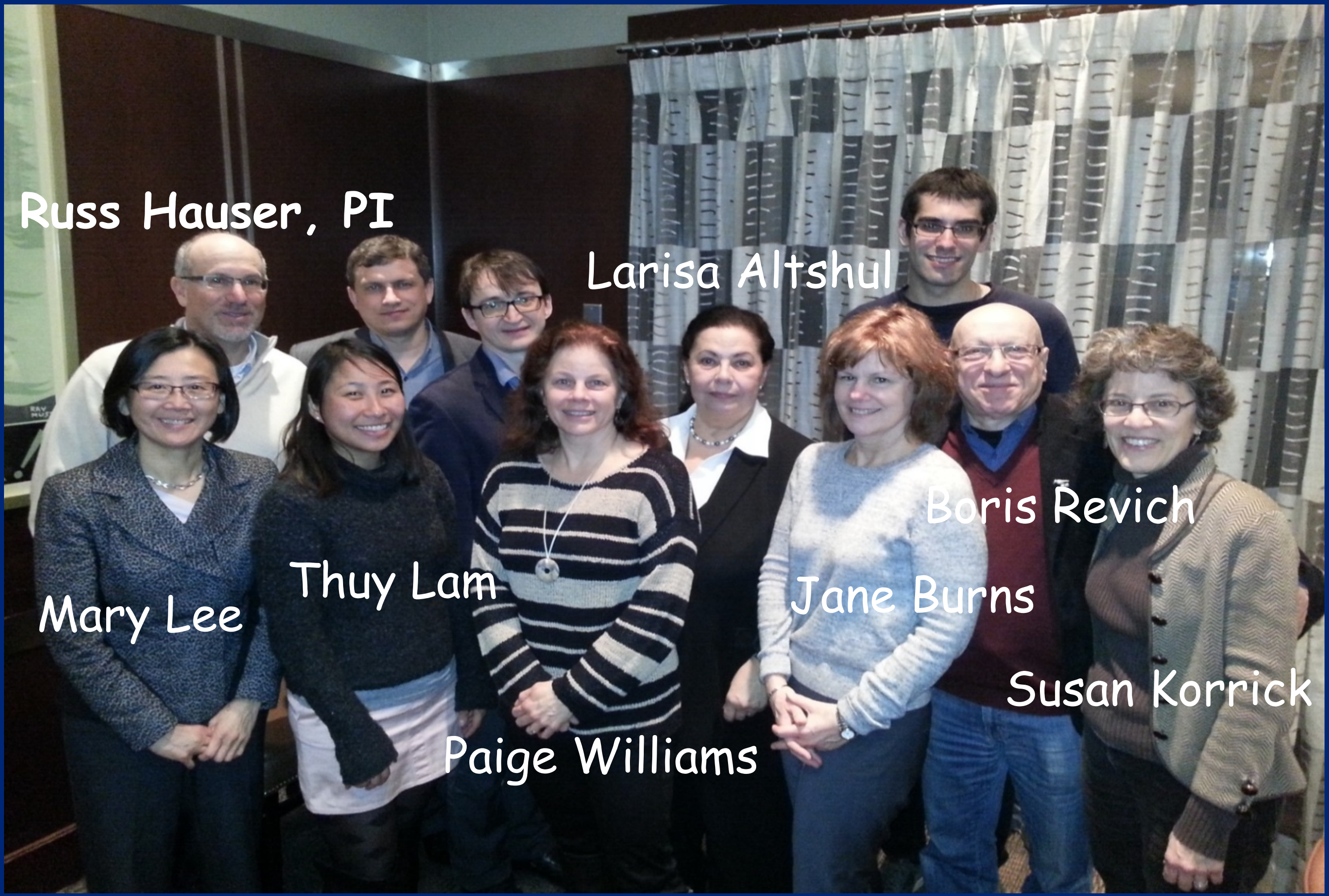
  - RRBS for whole cohort, n=217 (for n=51 done)
  - sncRNA in sperm and leukocytes (n=51) RSF #18-15-00202
  - Association of exposure, DNA methylation, sncRNA and semen quality

Key findings and conclusions

- Higher peripubertal serum concentrations of TCDD was associated with lower semen parameters 10 years later at age 18 yrs[1]
  - sperm concentration; - total sperm count; - total motile sperm count
- WGBS of sperm in young adults was conducted to examine associations with serum dioxin concentrations at 8-9 years
- Mean methylation across all CpG sites was lower in those with highest vs lowest TCDD concentrations
- 52 DMRs associated with peripubertal serum TCDD concentration[2]
- Estrogen receptor α (ESR1) is the central regulator under top scored function
- **First human study to show the association of the peripubertal environmental exposures with subsequent semen quality and sperm DNA methylation in humans**

[1]Mínguez-Alarcón et al, 2017; [2] Pilsner et al, 2018

Acknowledgements



Lidia Minguez-Alarcon

- Russian Children's Study – design, cohort enrollment and annual follow-up from 2003

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 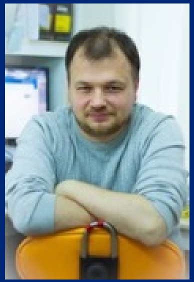   

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  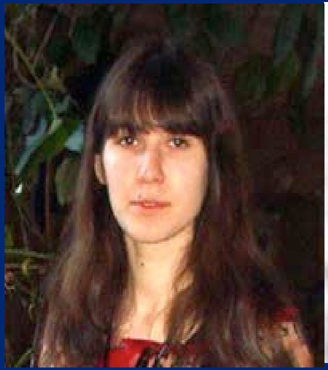   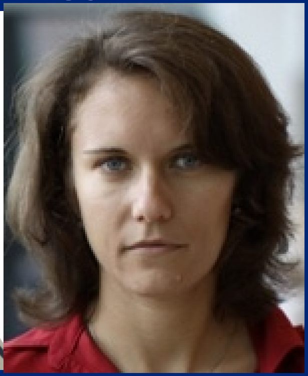

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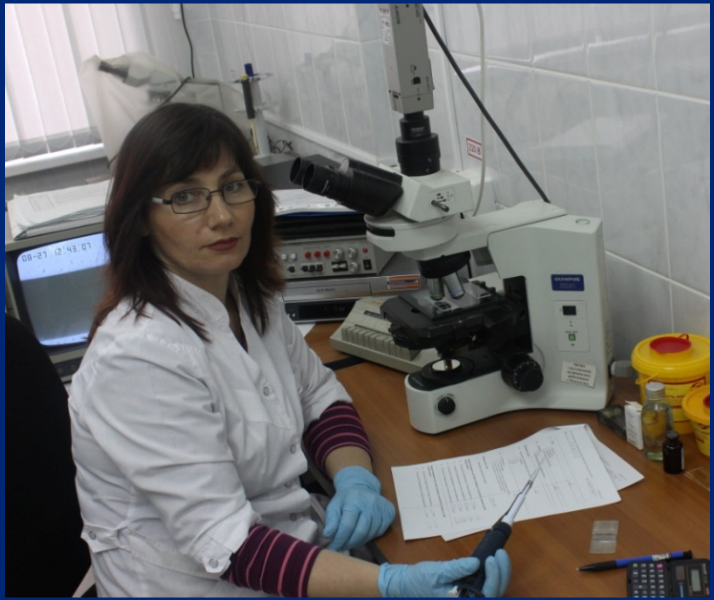
Study participants

Staff of Chapaevsk Medical Association (15 people)



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