RÉPUBLIQUE FRANÇAISE
Liberté Égalité Fraternité

INSTITUT NATIONAL DU CANCER

Spermatogenèse in vitro: sommes nous proche d'une application clinique?

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Du don à la vie

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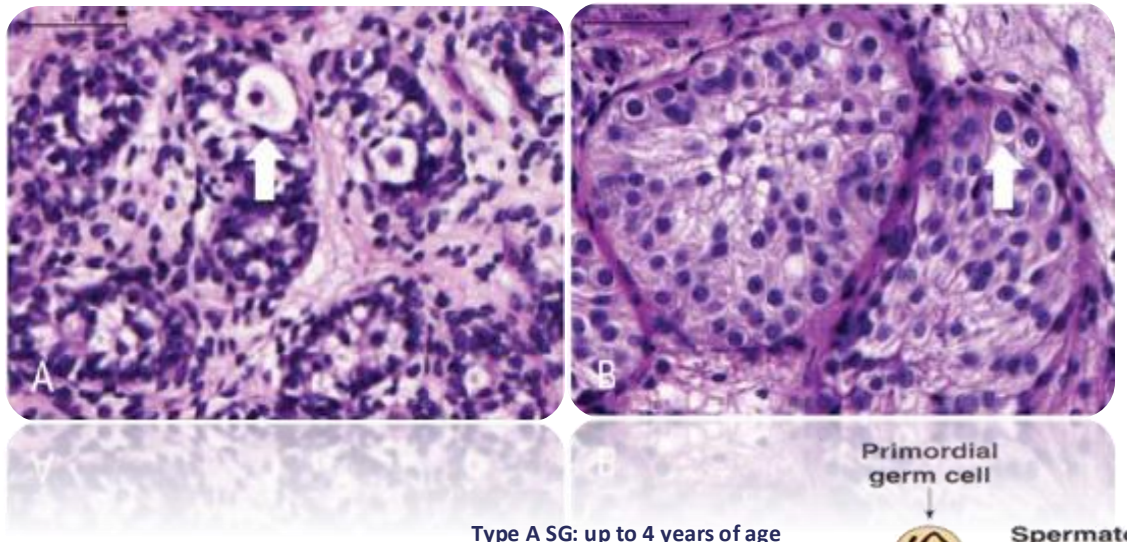
SÉMINAIRE RECHERCHE EN PRÉSERVATION DE LA FERTILITÉ CHEZ LES PATIENTS ATTEINTS D'UN CANCER

Introduction

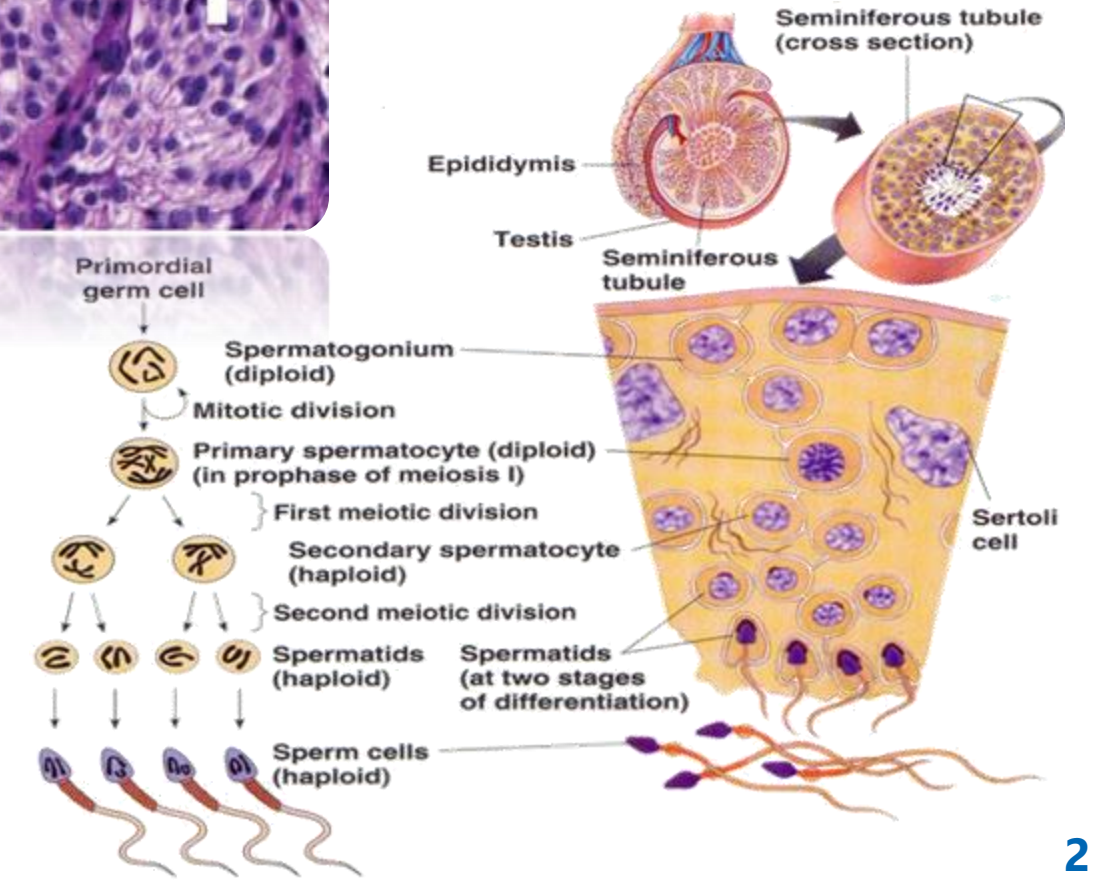
SSC cryopreservation is the only option for FP before puberty



Type A SG: up to 4 years of age
Type B SG: from 4 years of age, increasing up to 9 years of age

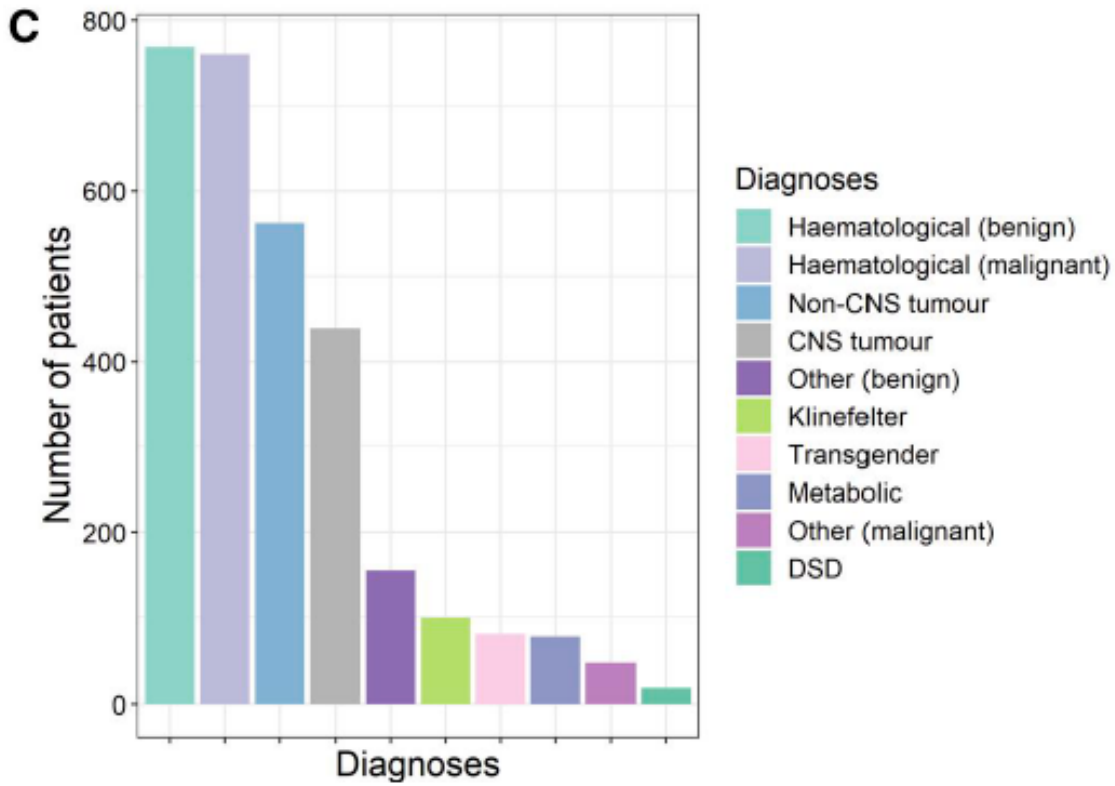
Spermatocytes: from puberty

spermiogenesis



Source: Andrology: Eberhard Nieschlag, Male Reproductive Health and Dysfunction; October 27, 2000

Introduction
Indications of ITT cryopreservation

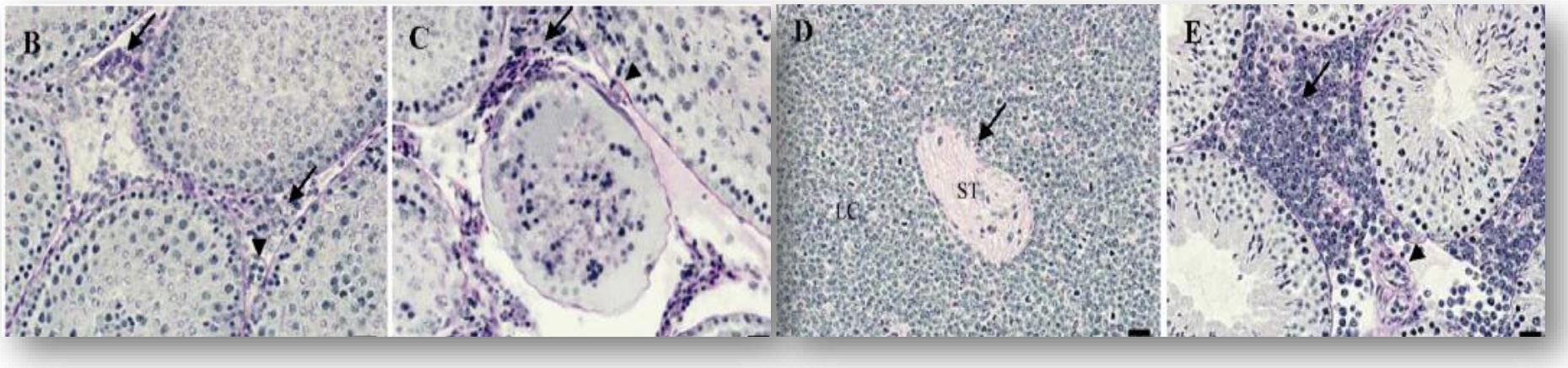


Breakdown of diagnoses of patients undergoing testicular tissue cryopreservation. CNS: central nervous system; DSD: disorder of sex development.

Duffin et al., 2024

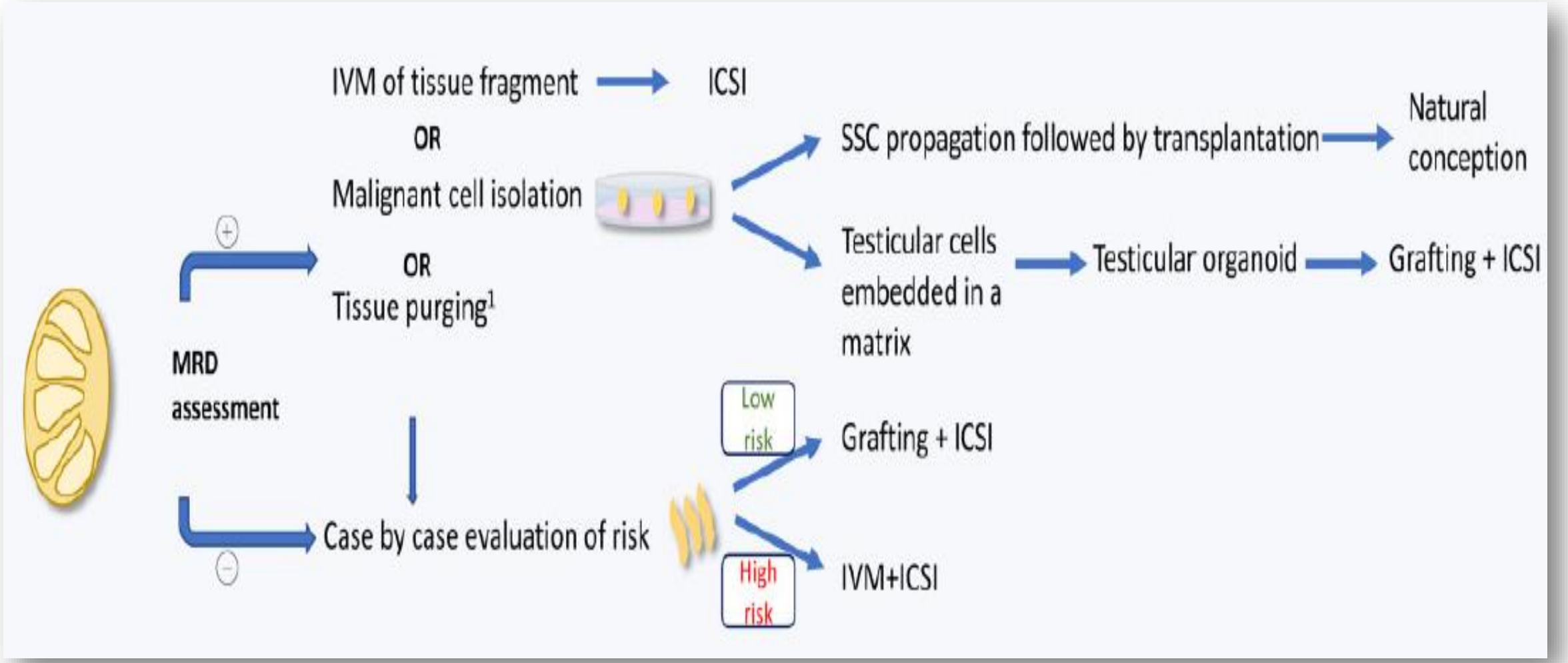
Introduction

- Risks of cancer cell contamination in hematological cancers
  - Malignant cells contamination of testes in cases of leukemias and lymphomas: 37% *(Kourta et al., 2023)*
  - As few as 20 leukemic cells injected into a testis can induce disease relapse *(Jahnukainen et al., 2001)*



Agressive proliferation of leukemic cells after cryopreservation
Tumor growth without potential to differentiate germ cells into gametes *(Hou et al., 2007)*

Introduction
Options for fertility restoration in malignant diseases



Kourta et al., 2023

Introduction

Cancer cell decontamination with a view to SSCs transplantation

Reference	Species	Cell-sorting technique	Markers	Evaluation after cell sorting	Outcome (% of residual contamination/ number of contaminated samples or mice)
Fujita et al., 2006	Human	FACS	MCH cl I $^{-}$ CD45^{-}	RT-PCR for germ cell markers (DAZL, HIWI, VASA, NANOG, STELLAR, OCT4)	1.45% K562 cells (CML), 0% K562 cells after IFγ (for induction of MCH cl I)
Geens et al., 2007	Human	FACS	H2Kb^{-} (MCH cl I)	FACS; In vitro culture; PCR for B cell receptor	0.58% SB^{+} cells 1/11 contaminated samples
Hermann et al., 2011	Non-human primates	FACS	CD90^{+}/CD45^{-} --- SD replicates	Xenografts in mice + epifluorescent microscopy --- Post-sorting purity	0,1% contamination + tumors --- No tumor
Geens et al., 2011	Human	Culture + MACS + Matrix adhesion-based selection	CD49F+ HLA cl 1^{-}	FACS PCR after purging	B- cell receptor in SB cells in 5/5 (0.9-4.6%)
Dovey et al., 2013	Human	FACS	EpCAM^{+}/CD49^{-}/HLA-ABC^{-}	Xenografts in mice Postsorting purity	0% contamination; (vs 23-55% for cancer cell fraction) 98.8-99.8%

MCH cl I: major histocompatibility complex class I (marker of somatic cells); α6 integrin: marker of SSCs; CD45: surface marker of leukemic cells; IFγ: interferon-γ; CML: chronic myelogenous leukemia; SD: singlet discrimination; CD90: Thy-1 cell surface antigen.

→ Post-sorting purity checks are required to confirm elimination of malignant cells

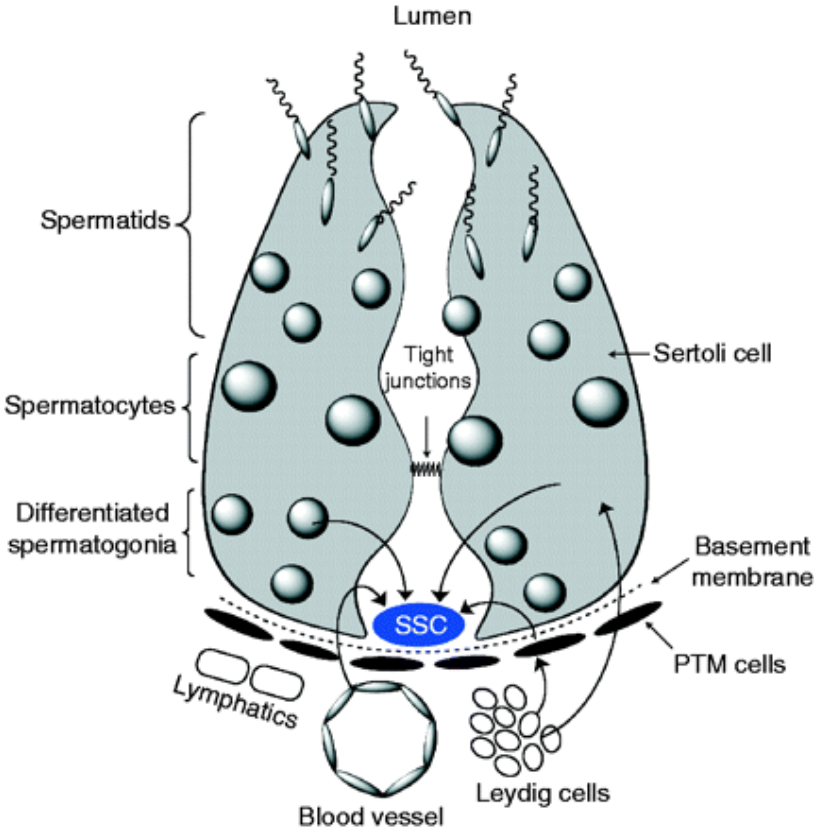
Adapted from de Michele, Vermeulen, Wyns, Current opinion, 2017

Introduction
Indications of IVM

- Malignant conditions with risk of cancer cell contamination of the testes
- Benign conditions with a dysfunctional testicular somatic compartment
- Transgender patients to avoid exposure to elevated testosterone if *in vivo* use of their tissue

In vitro maturation……challenges

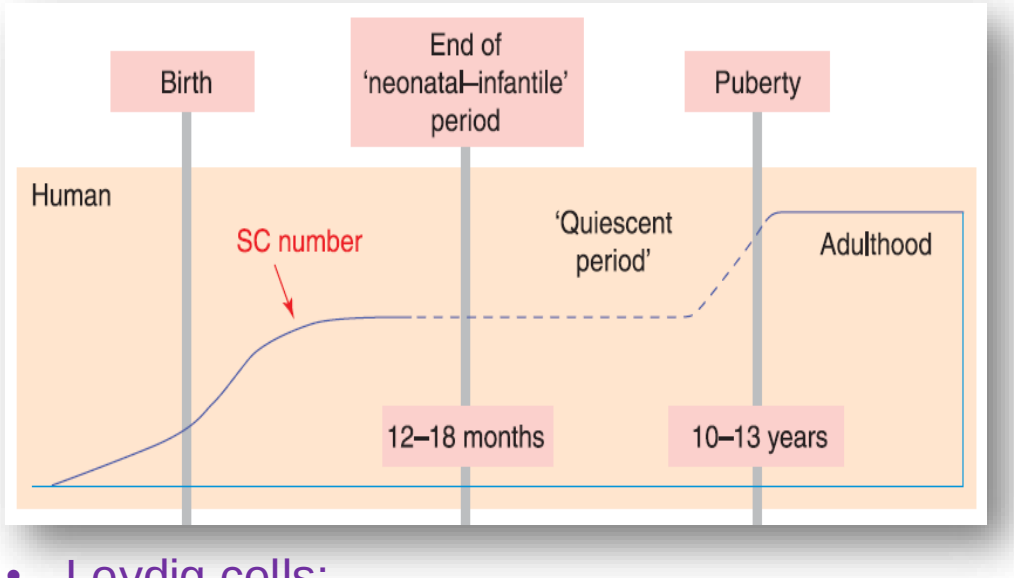
➢ Reproducing the SSC niche maturation process



Architectural and molecular support

- Sertoli cells:

Prepubertal proliferation
Peripubertal proliferation arrest → maturation



- Leydig cells:

Testosterone production

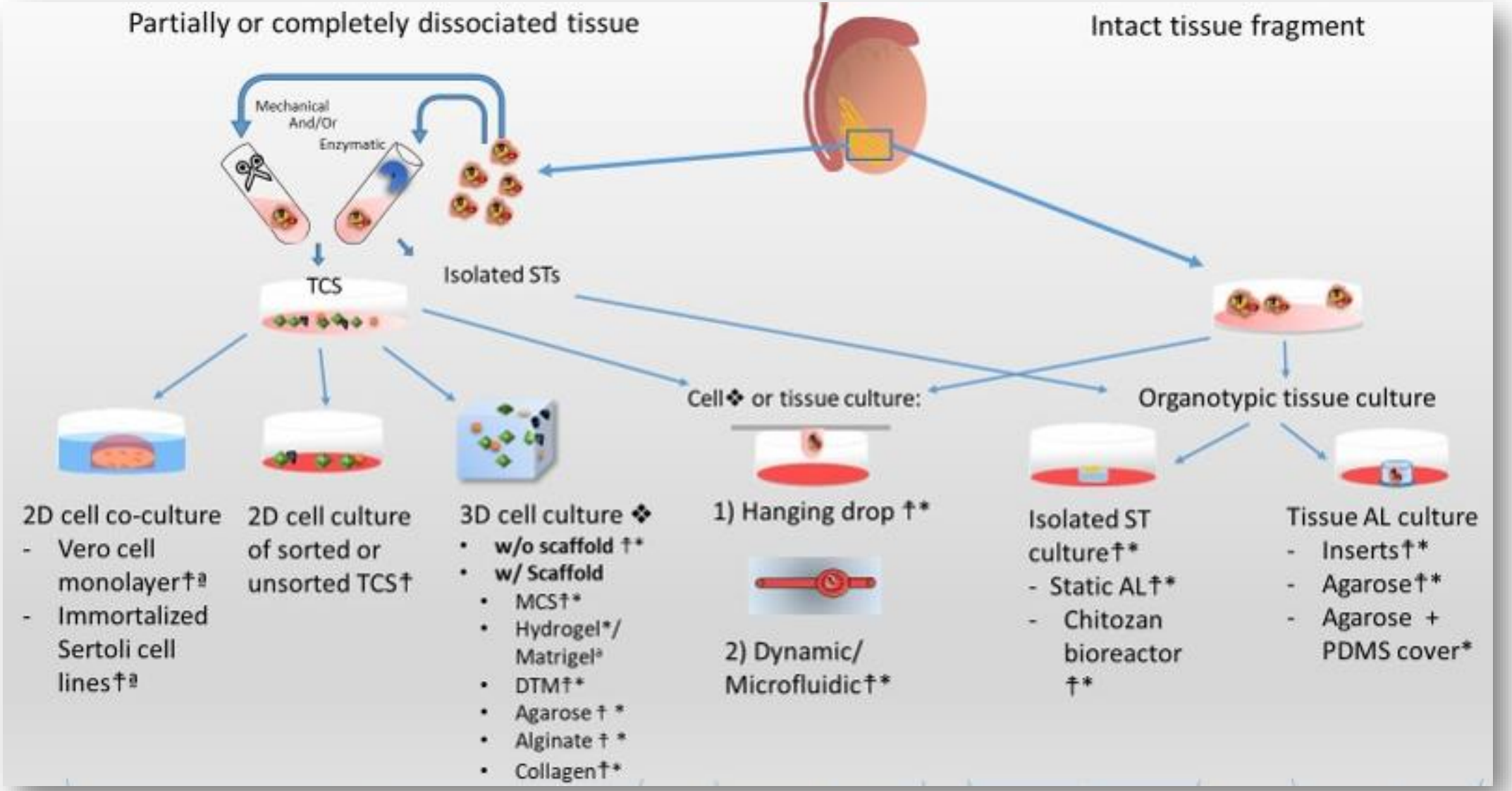
Caires et al., 2010 Sharpe et al., 2003

In vitro maturation……challenges

- Reproducing the spermatogenic process

Culture systems	Steps to develop functional gametes
ITT: organotypic (3D)	• Define clinical grade medium • Achieve IVM from the SSC stage • Assess the quality of gametes • Prove the fertilization potential
SSC: cell culture (2D/3D)	
Organoids: reflects the original testis micro-anatomy	

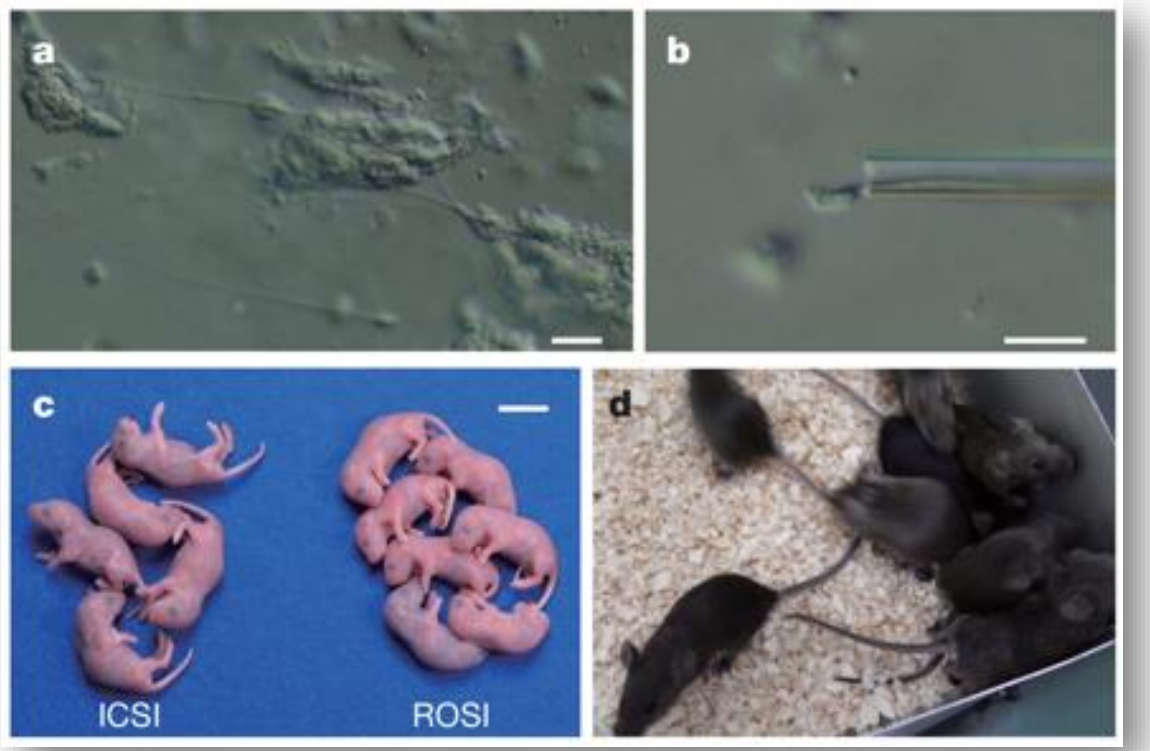
In vitro maturation: methods



Achievements in animals

Mice offspring with reproductive competence

- Neonatal tissue
- Organotypic culture at gas-liquid interphase
- KSR

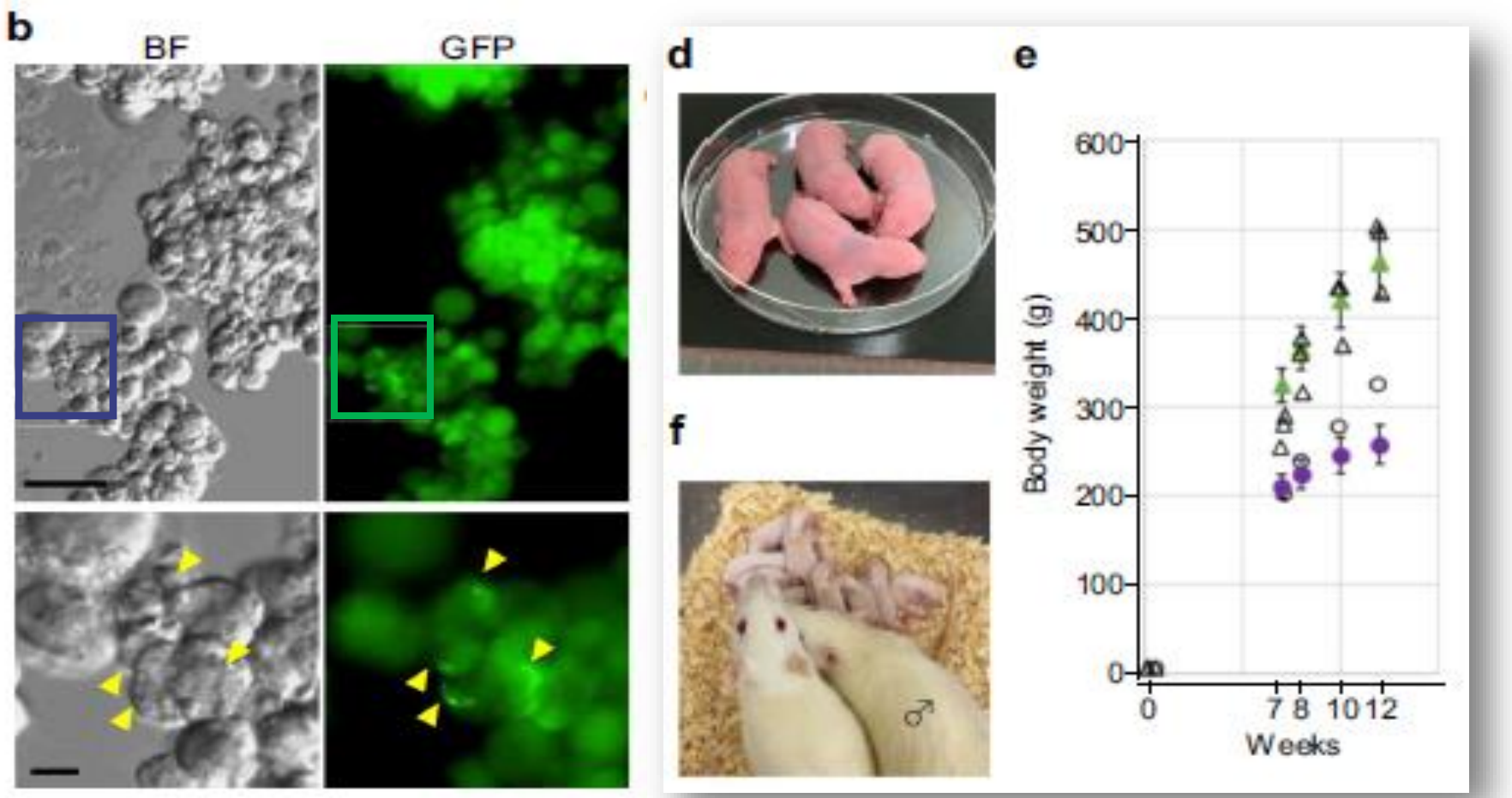


Sato et al., 2011

Achievements in animals

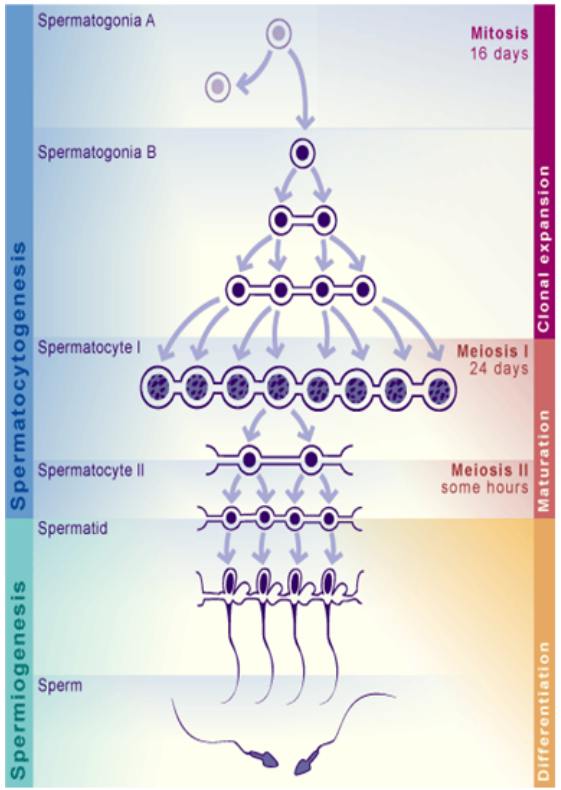
Rat offspring

- Organotypic culture under PC chip -47 days
- ROSI



Matsumura et al., 2023

IVM (human adult testicular tissue)



Normal mature tissue

Cryptorchid mature tissue

Tissue from transexual men after 2 years - hormonal therapy

Lee J 2007 12 days

Lee D 2006 6 weeks

Roulet 2006 16 days

Tesarik 1998 48h

Yang 2014 10 days

Perrard 2016

3D

Organotypic culture

Partial digested tissue

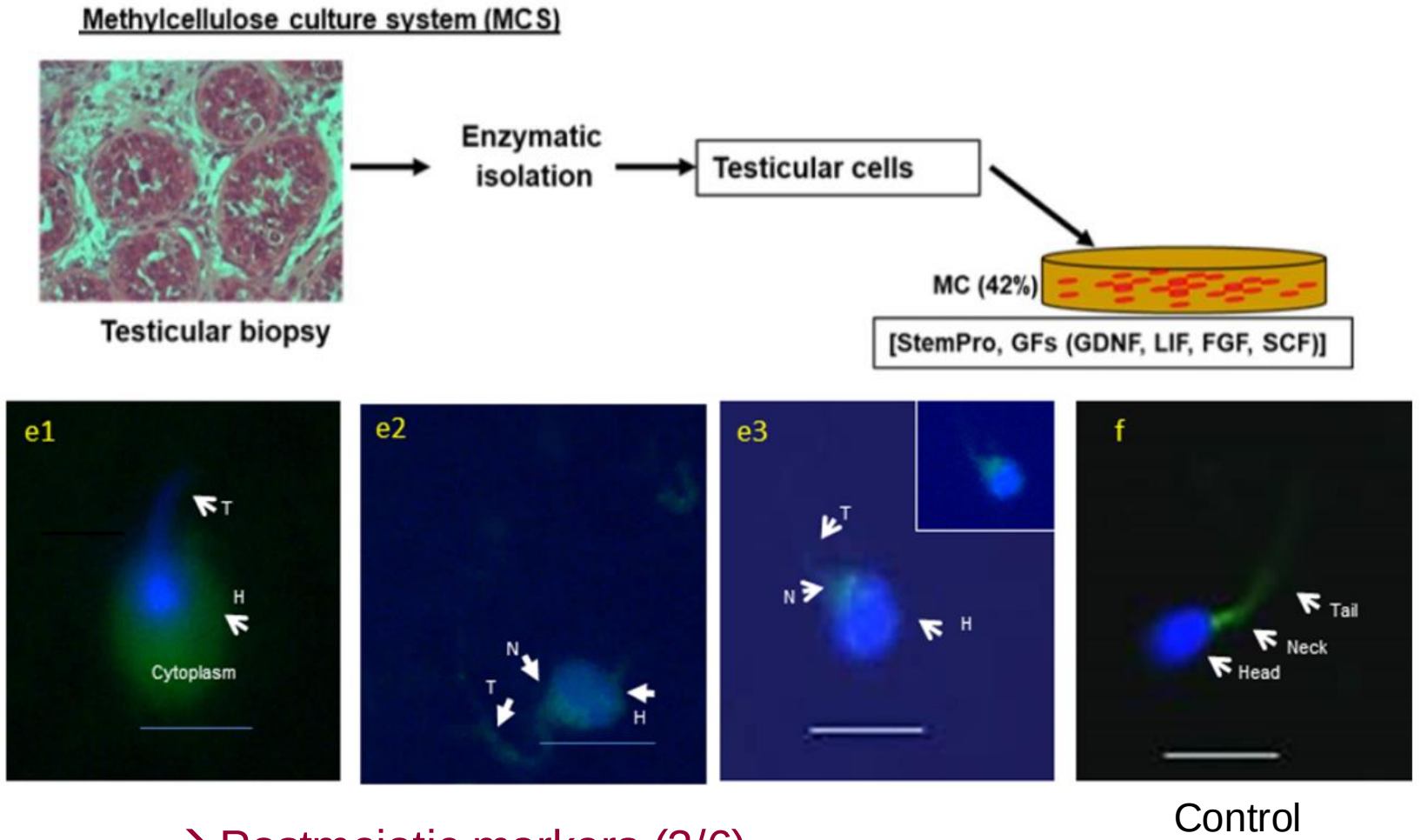
Cellular suspensions

Isolated seminiferous tubules segments

Sun 2018 20 days Fertilization of mouse oocytes

IVM (human prepubertal testicular cells)

3D culture
5-15 weeks



Control

→ Postmeiotic markers (2/6)
→ Sperm-like cells (1/6)

Abofoul-Azab et al., 2018

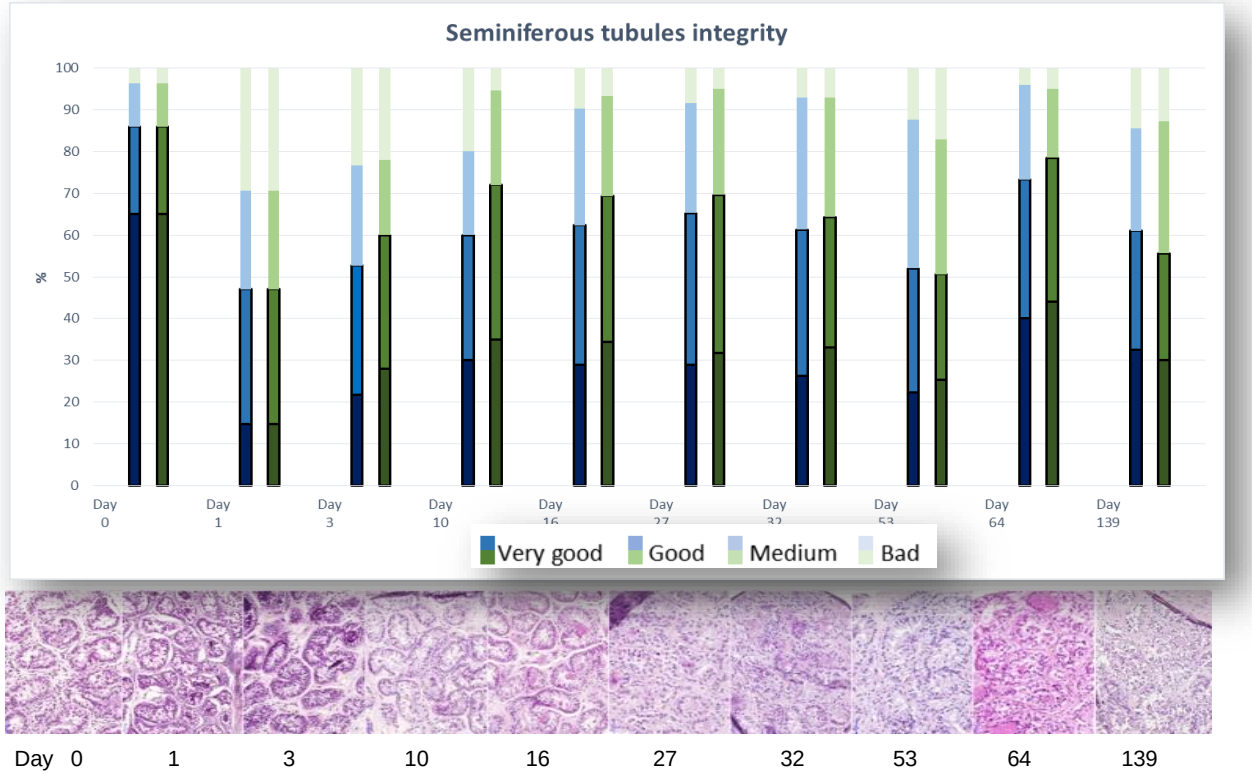
IVM (human prepubertal testicular tissue)

Studies with human prepubertal tissue	Culture approach	Culture outcome (germ cell differentiation)	Evidenced by
De Michele et al., 2017	Organotypic	Spermatogonia	IHC
De Michele et al., 2018	Organotypic	Round spermatids	IHC + CISH (haploidy)
Medrano et al., 2018	Organotypic	Spermatocytes	ICH + qPCR
Portela et al., 2019	Organotypic	Spermatogonia	IHC
Younis et al., 2023	Organotypic	Spermatocytes	IHC
Aden et al., 2023	Organotypic	Spermatogonia	IHC

IVM (human prepubertal testicular tissue)

- Long term organotypic culture

Tubules' integrity



- Millicell inserts
- Air-liquid interface
- 34°C-5% $C0^2$
- DMEM/F12-HSA 20%
- FSH 50 IU/l

Testosterone ($7\ 10^{-9}$ M) hCG (1 IU/l)

→ STs are well preserved during the culture
→ No difference between the two culture media

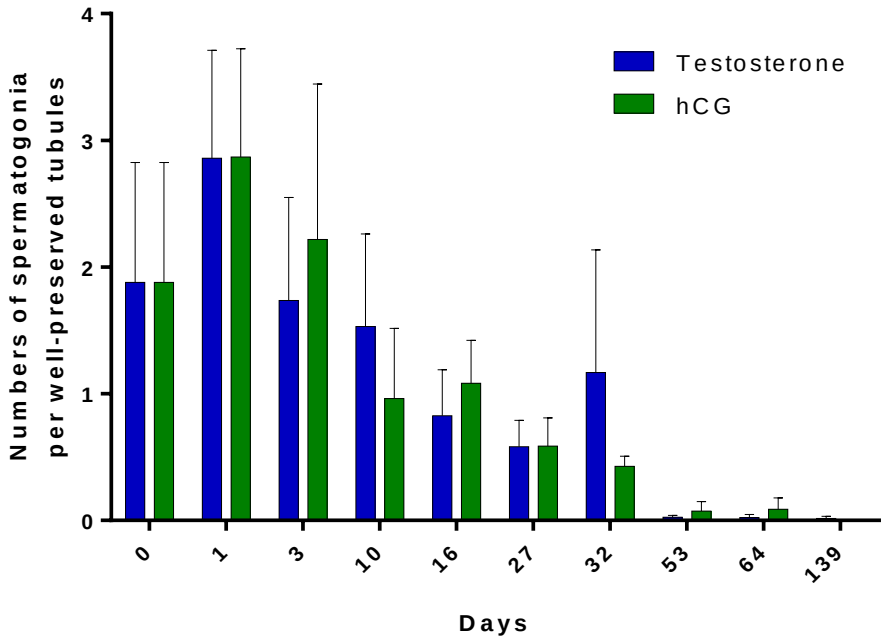
De Michele et al., 2017

IVM (human prepubertal testicular tissue)

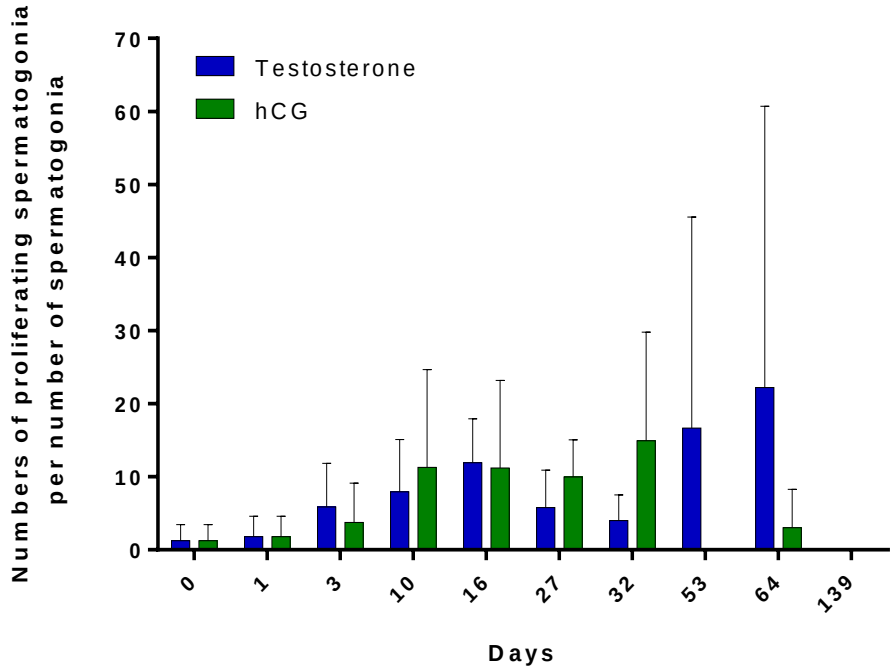
- Long term organotypic culture

Spermatogonial cells

survival



proliferation capacity



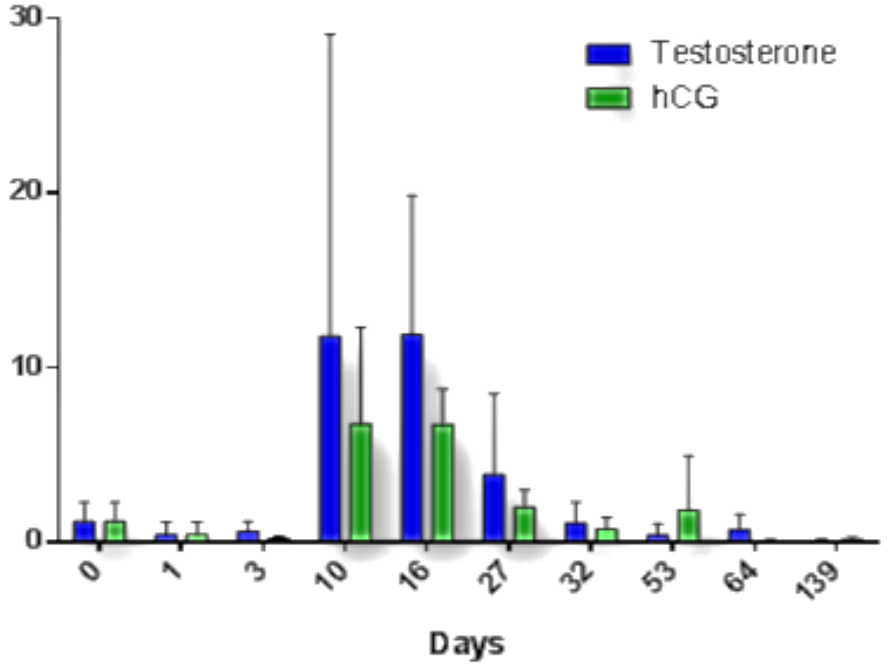
De Michele et al., 2017

IVM (human prepubertal testicular tissue)

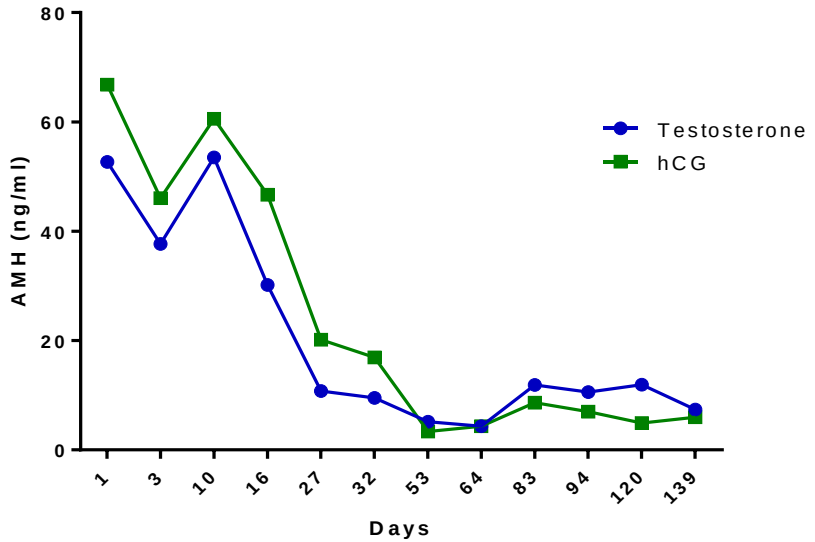
- Long term organotypic culture

Sertoli cell maturation

Proliferation



AMH secretion

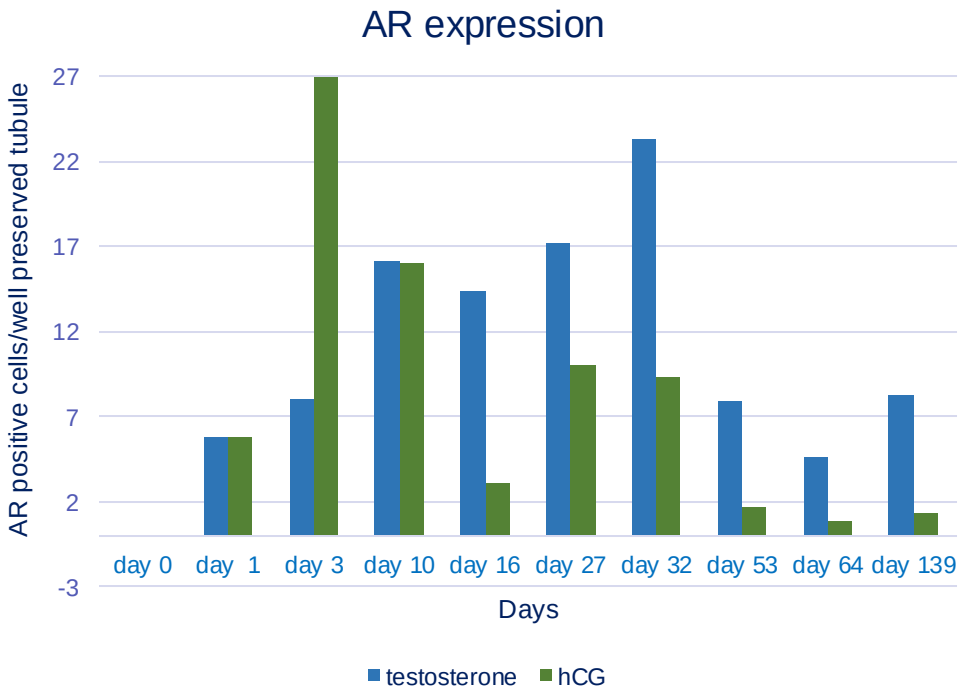


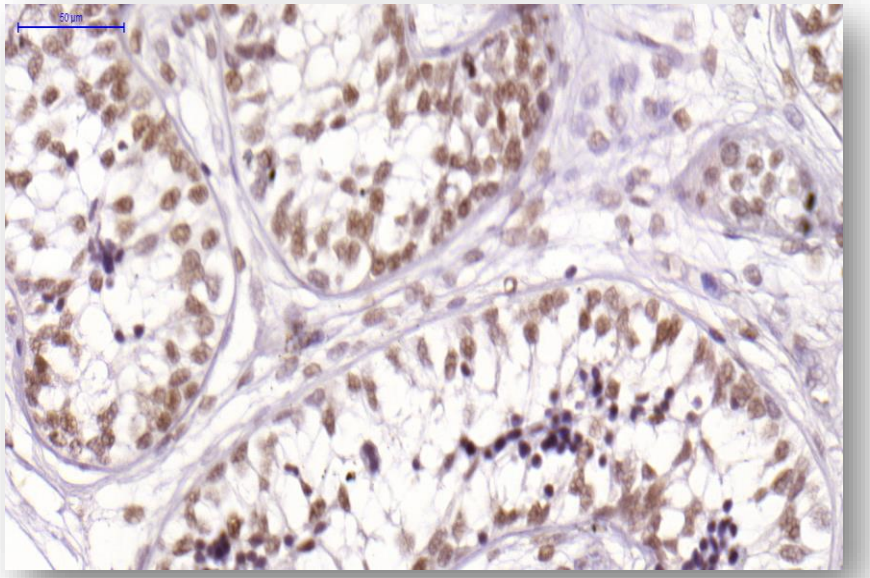
De Michele et al., 2017

IVM (human prepubertal testicular tissue)

- Long term organotypic culture

Sertoli cell maturation: androgen receptor expression





2-year old boy

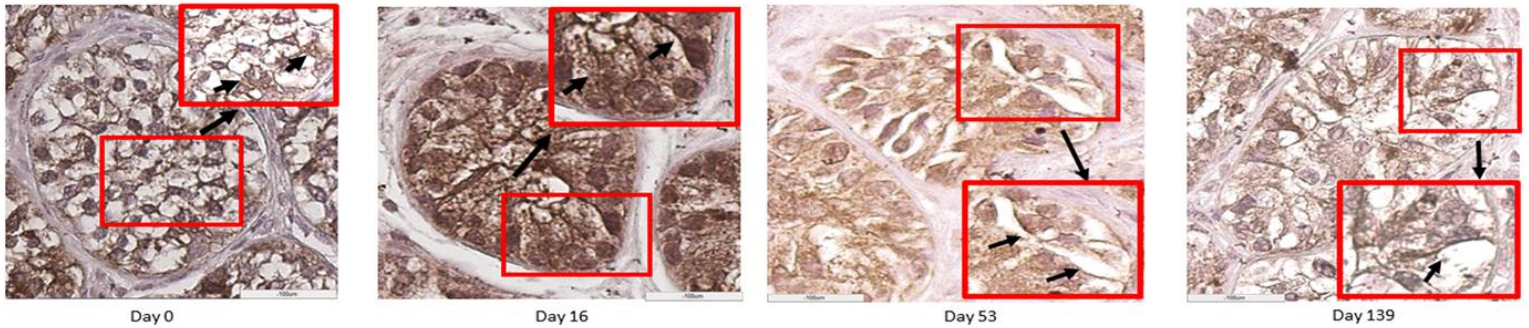
De Michele et al., 2017

IVM (human prepubertal testicular tissue)

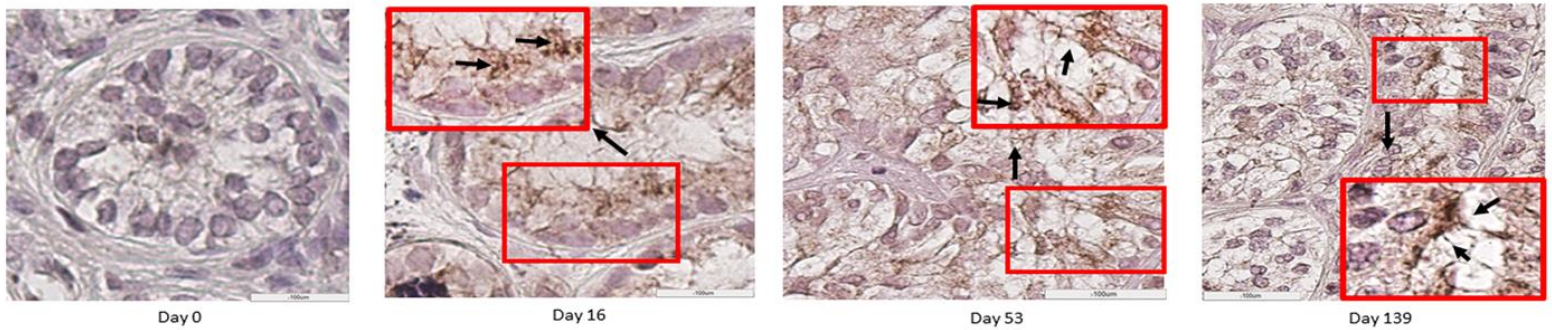
- Long term organotypic culture

Blood-testis barrier formation

Claudin 11 (tight junction-between Sertoli cells and between Sertoli and germ cells)



Connexin 43 (gap junction-communication between Sertoli cells and between Sertoli and germ cells)



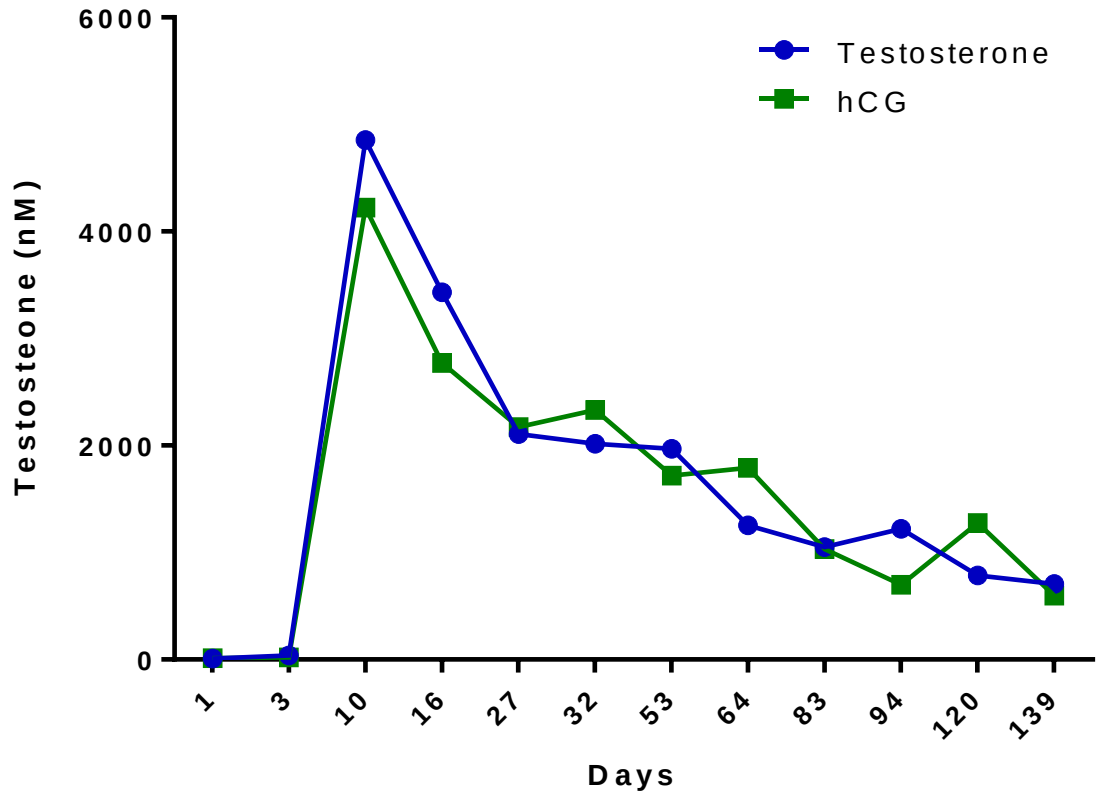
→ Claudin 11: constant expression in culture
→ Connexin 43: expression from day 16 of culture

De Michele et al., 2018

IVM (human prepubertal testicular tissue)

- Long term organotypic culture

Leydig cells survival and functionality

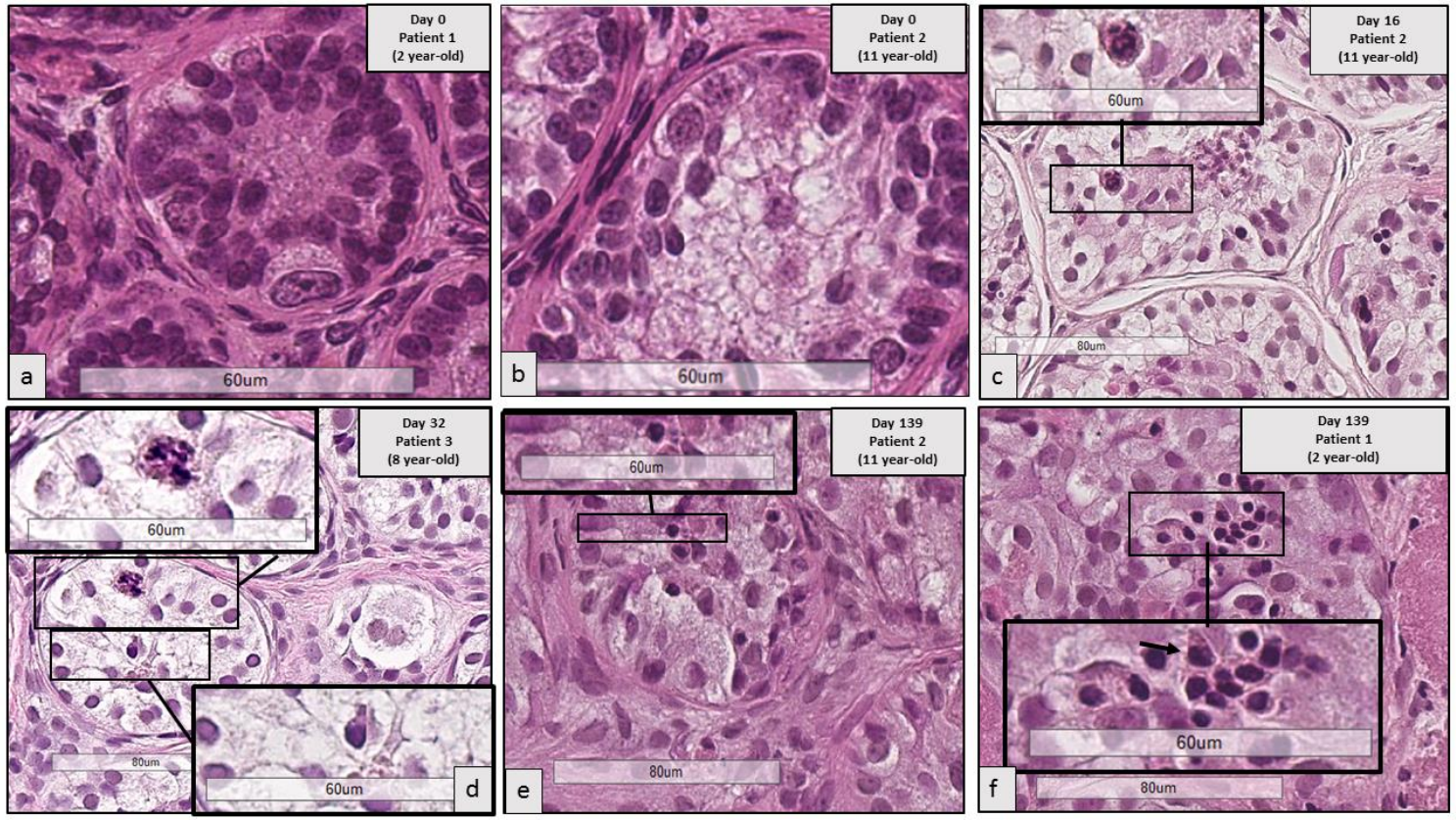


De Michele et al., 2017

IVM (human prepubertal testicular tissue)

- Long term organotypic culture

Germ cell differentiation up to haploid cells: KSR-FSH 5 IU/l



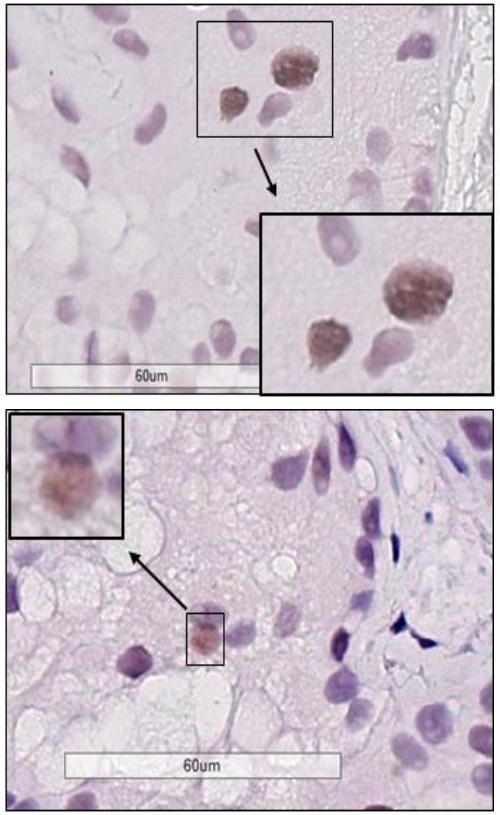
Presence of spermatocytes and round spermatids after 16 days of culture

De Michele et al., 2018

IVM (human prepubertal testicular tissue)

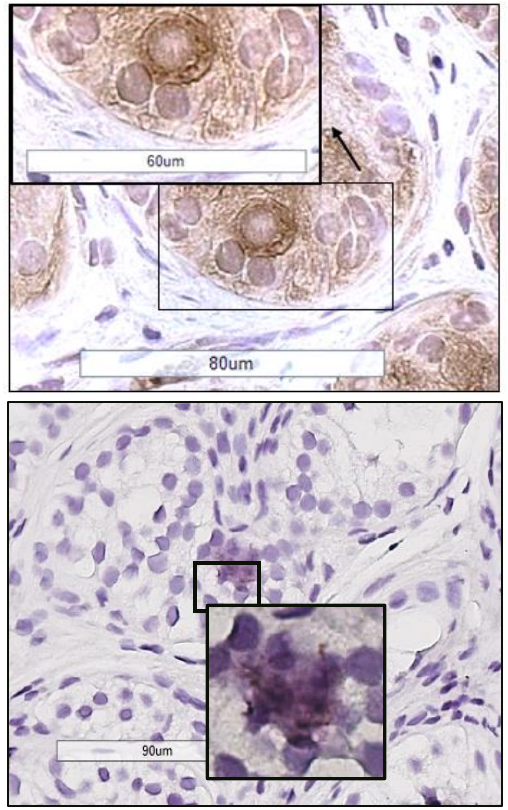
- Long term organotypic culture

Germ cell differentiation: meiotic ant postmeiotic cells



SYCP3
Patient 5 (12 years old)
Day 16 M1 (top) and 32 (bottom)

BOLL
Patient 1 (2 years old) Day 16 M1



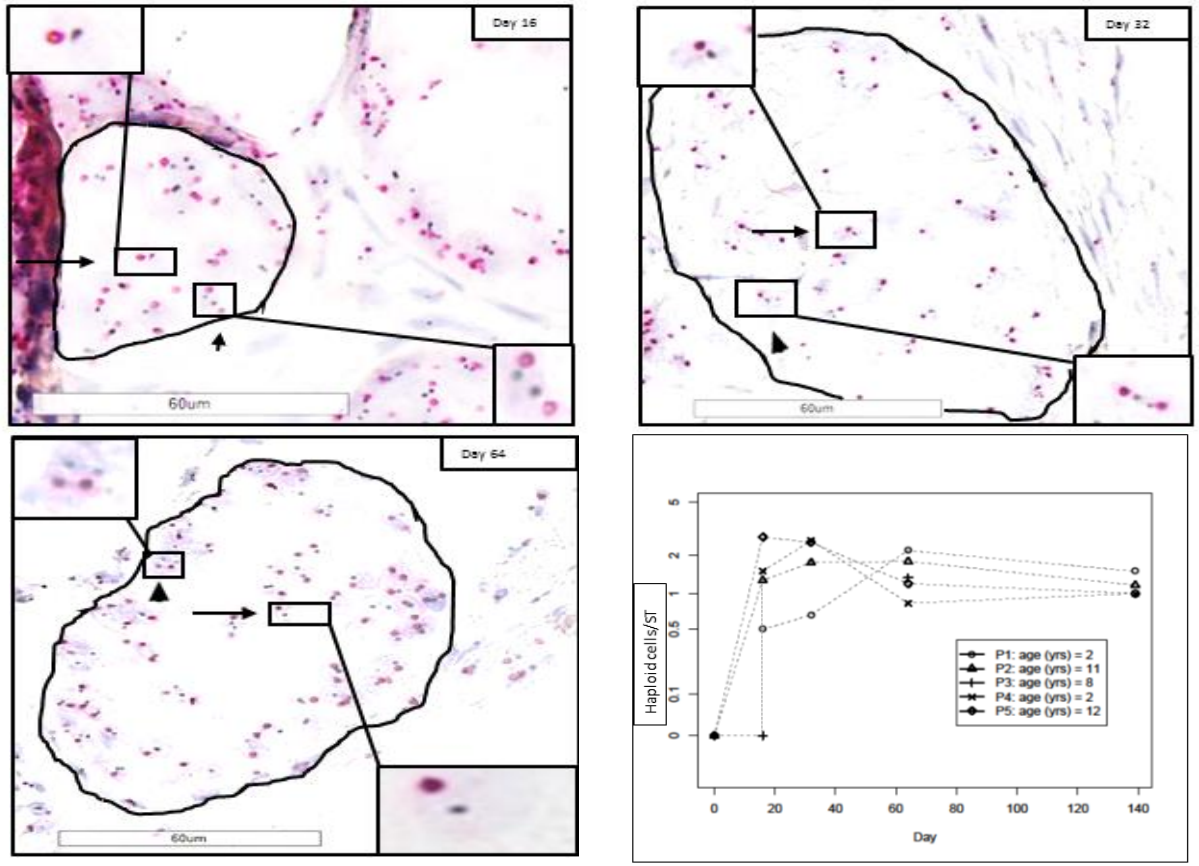
ACE
Patient 3 (8 years old)
Day 64 M2

De Michele et al., 2018

IVM (human prepubertal testicular tissue)

- Long term organotypic culture

Germ cell differentiation: haploid cells



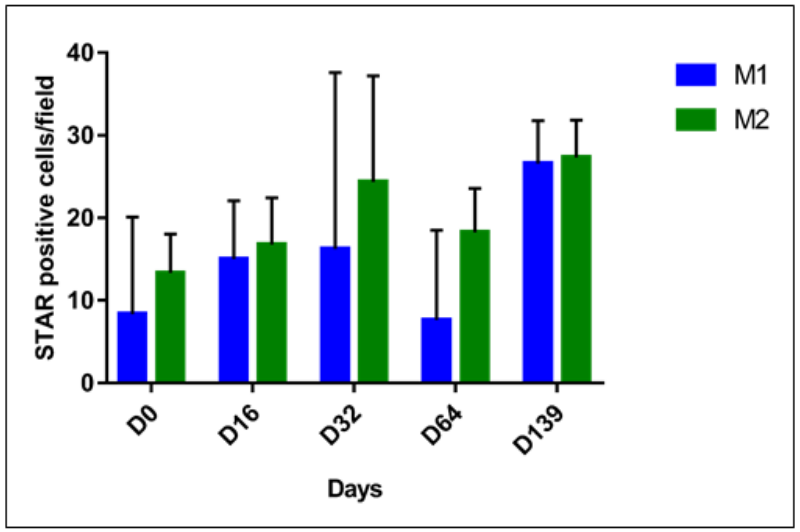
Red: chromosome 17
Black: HER 2

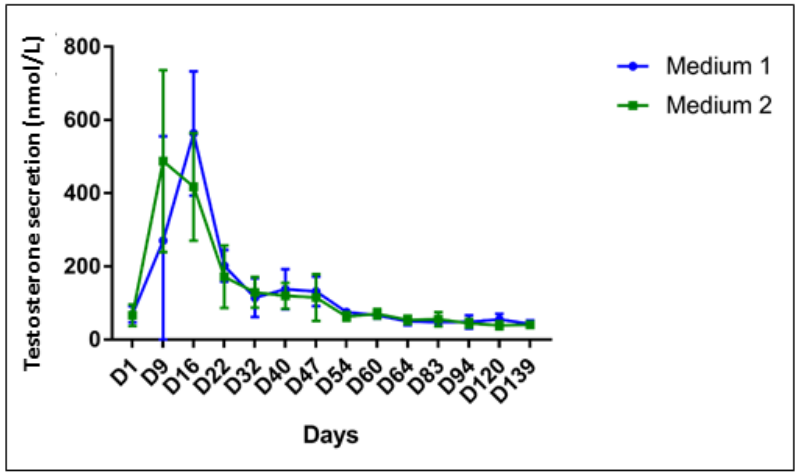
De Michele et al., 2018

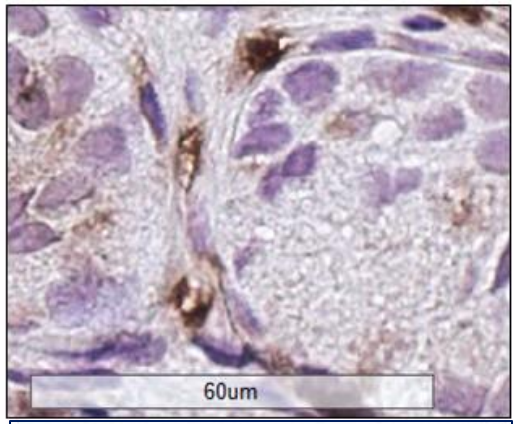
IVM (human prepubertal testicular tissue)

- Long term organotypic culture

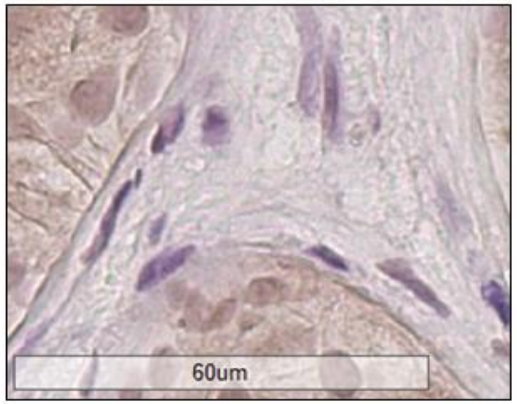
Leydig cell differentiation







STAR presence of interstitial staining



STAR absence of staining (negative control)

De Michele et al., 2018

Challenges before clinical application of IVM of prepubertal testicular tissue

Loss of germ cells

Incomplete maturation and characterization of germ cells

Very low efficiency

→ Understand reasons for germ cell loss

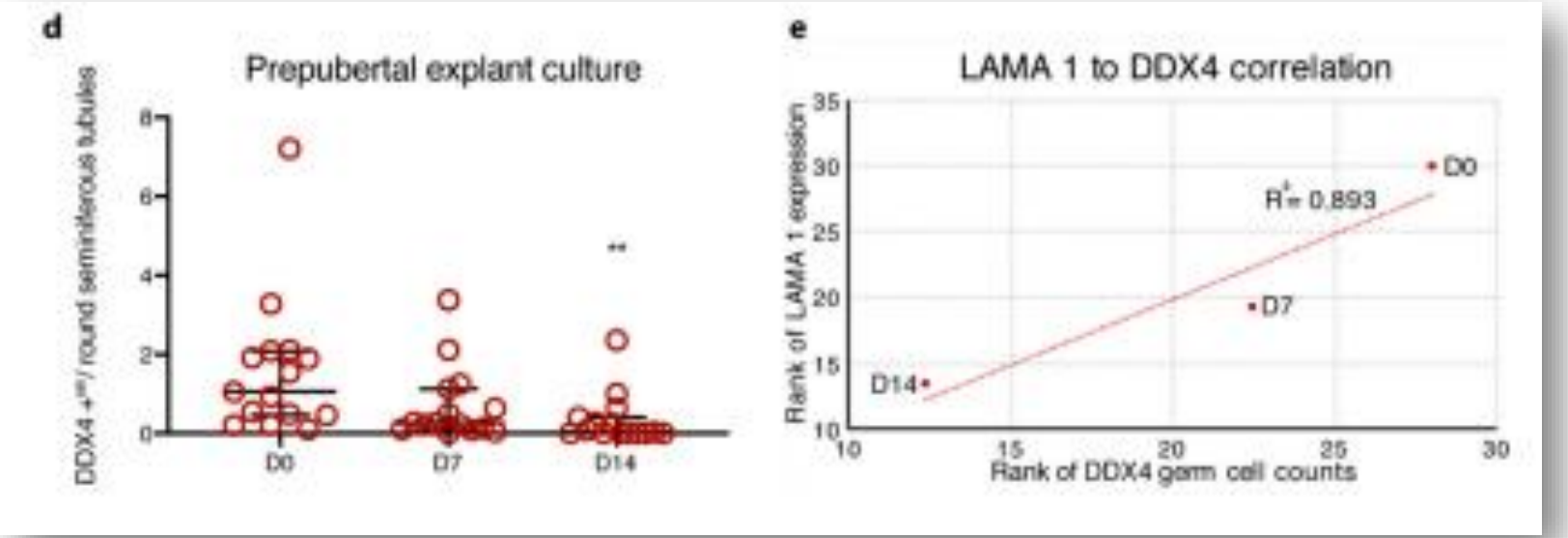
→ Compare culture systems

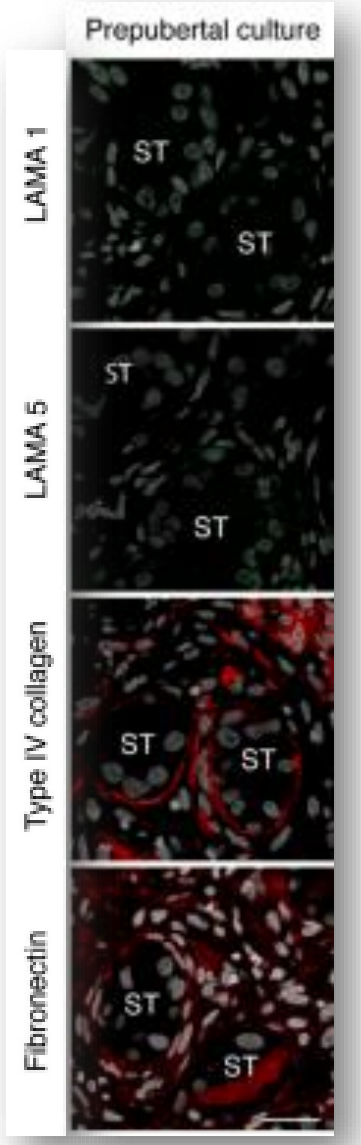
→ Improve culture media

→ Explore the need for individualization (pubertal stage)

Understand reasons for germ cell loss

Air-liquid interphase on agarose
35°C
KSR 10%

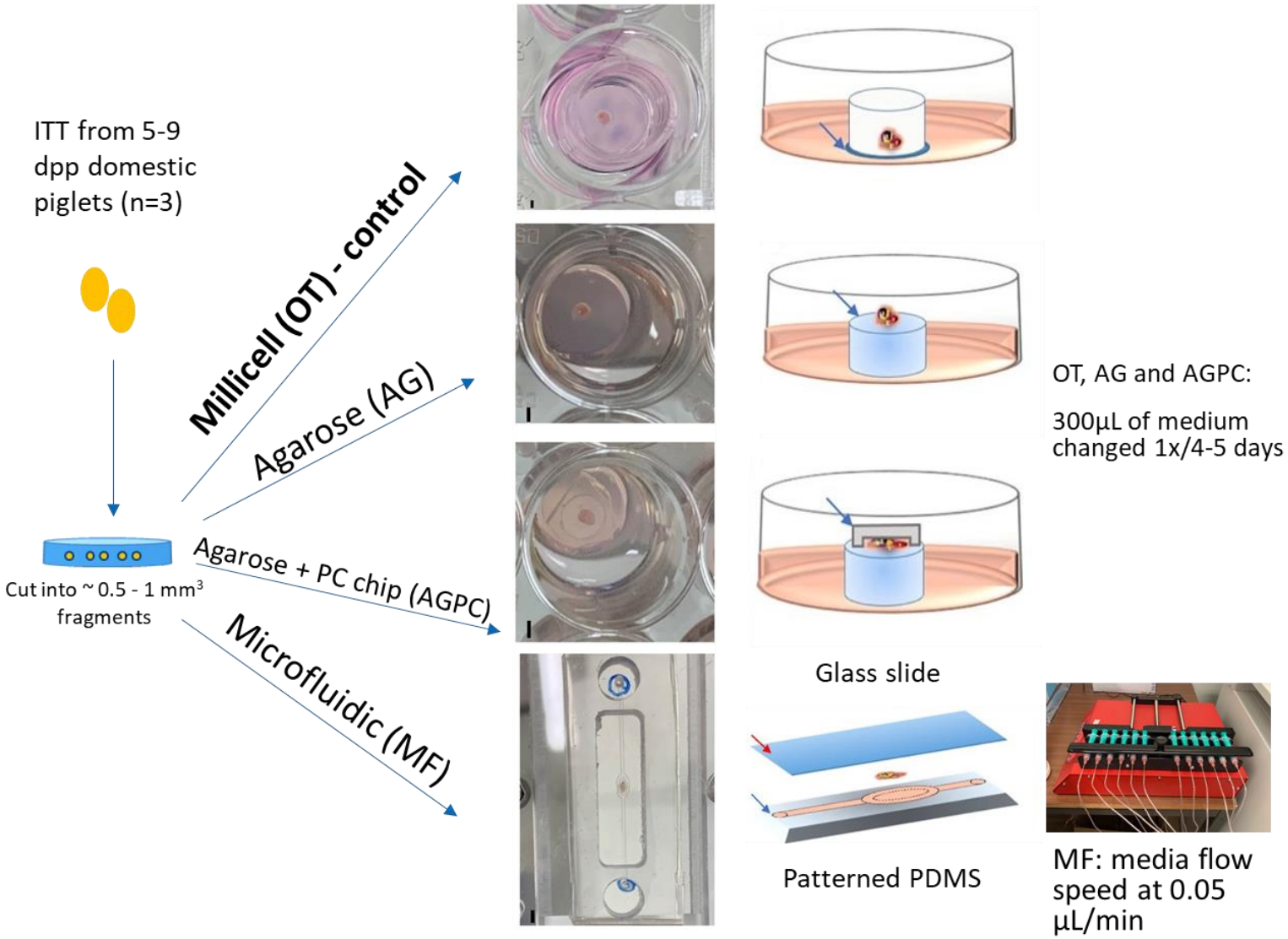




→ Loss of GCs and LAMA1 expression

Kurek et al., 2021

Comparing culture systems



Culture medium ***(de Michele et al., 2018):***

1- KSR CTS™ KnockOut™ SR XenoFree Medium 10% in CTS™ KnockOut™ DMEM/F-12
2- FSH 5 IU/L
3- Gentamycin + Ceftazidime

Culture conditions:
34°C in 5% CO_2

Culture duration :
Up to 30 days

Timepoints :
Days: 0, 5, 10, 20, 30

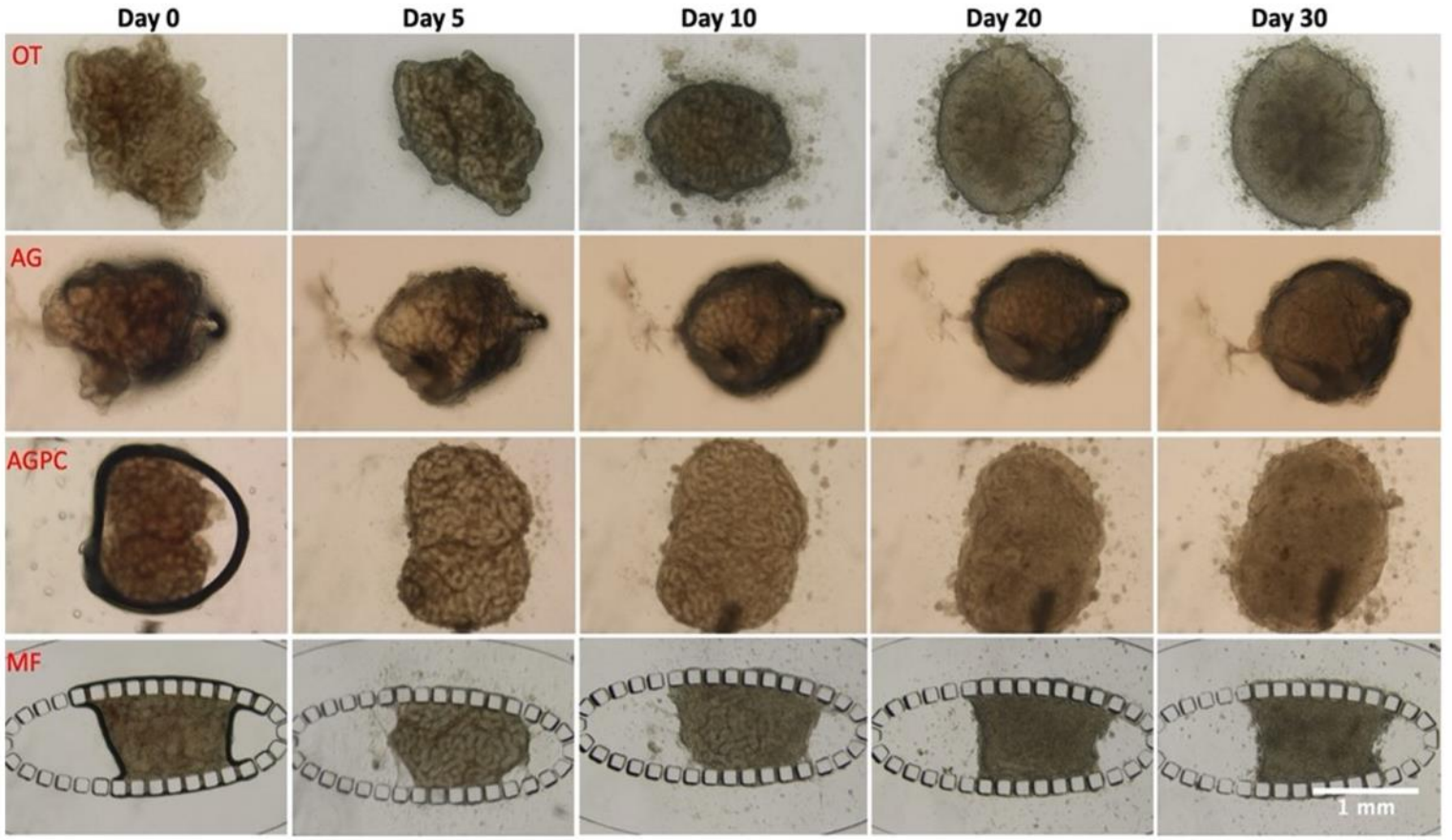
De Michele et al., 2018., Front Phys Komeya et al., 2016, Sci Rep.

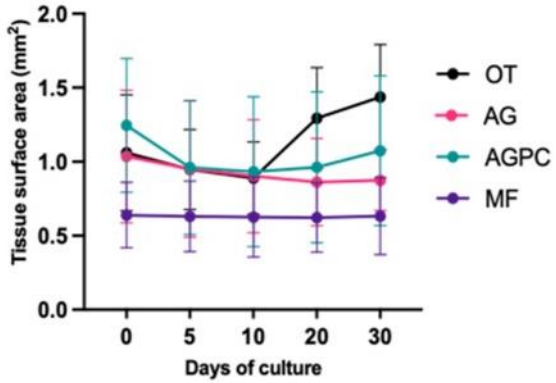
Kanbar et al., 2022 UV sterilization for PVC tubes and autoclave for the rest of the material used

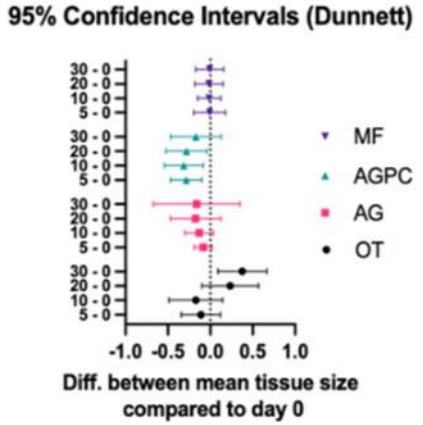
Comparing culture systems

IVM (porcine prepubertal testicular tissue)

➢Live imaging-serial tissue photographs







Kanbar et al., 2022

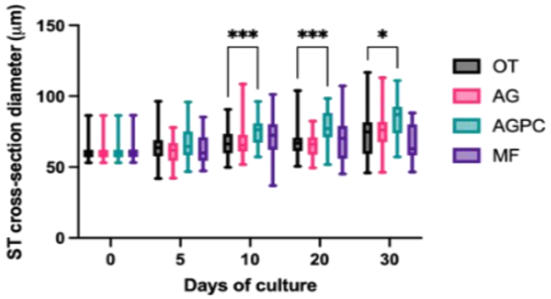
Control: OT and day 0

Comparing culture systems

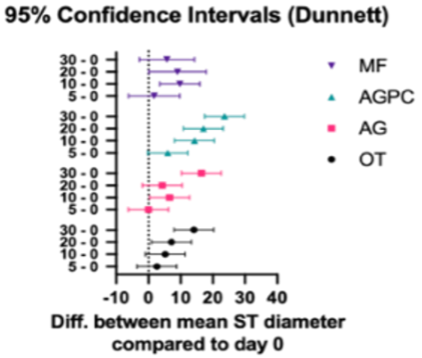
IVM (porcine prepubertal testicular tissue)

➢ Mean ST diameters, ST and tissue core integrity

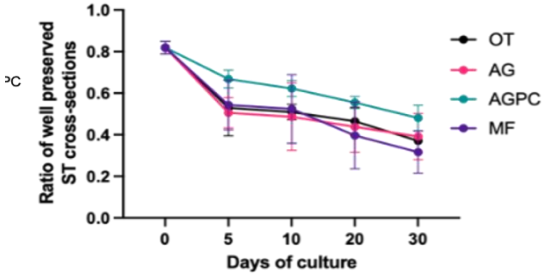
Mean ST diameter



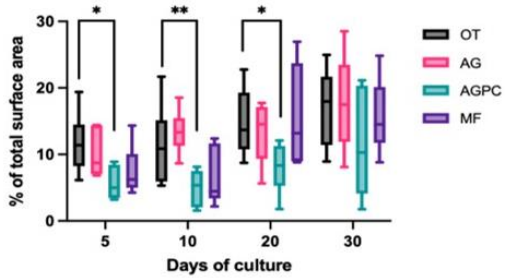
Mean ST diameter



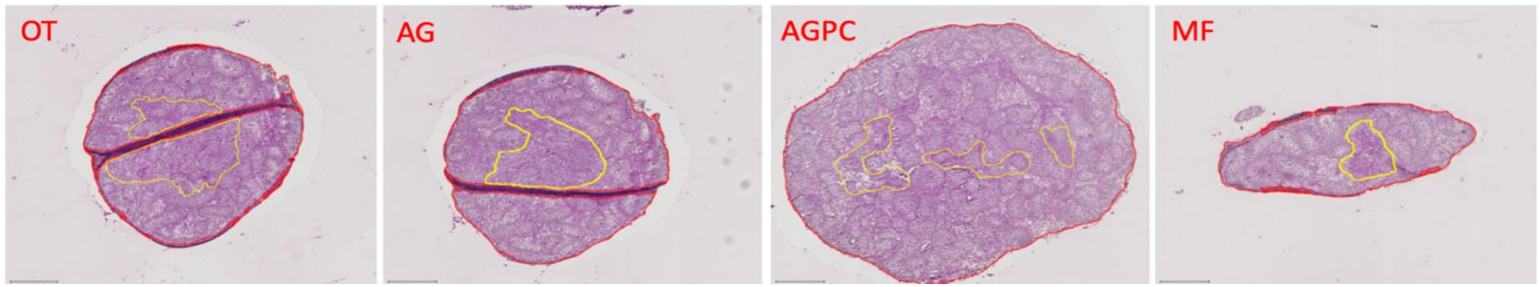
ST integrity



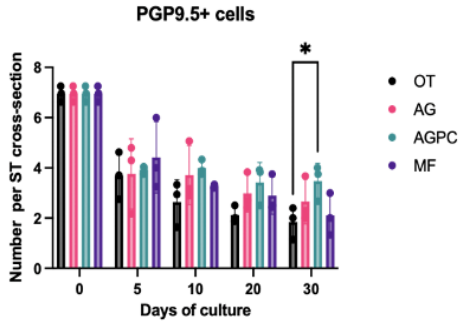
Tissue core degeneration



Statistically significant results were reported as ∗ ($p \leq 0.05$), ∗∗ ($p \leq 0.01$), ∗∗∗($p \leq 0.001$) and ∗∗∗∗ ($p \leq 0.0001$) on graphs



Undifferentiated spermatogonia



Kanbar et al., 2022

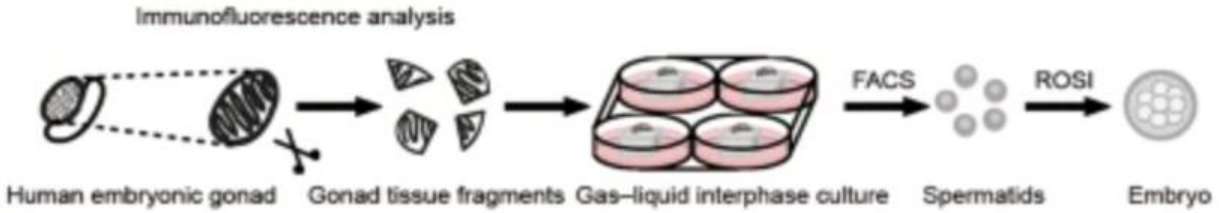
Improving culture media
High testosterone concentrations

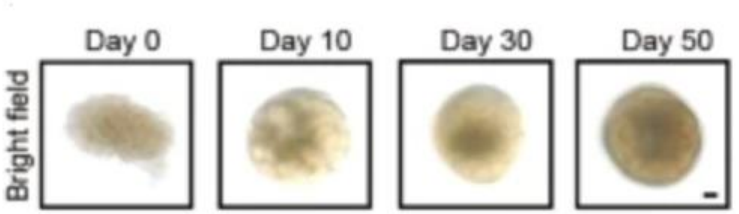
In vitro testicular organogenesis from human fetal gonads produces fertilization-competent spermatids

Yan Yuan, Laihua Li, Qing Cheng, Feiyang Diao, Qiao Zeng, Xiaoyu Yang, Yibo Wu, Hao Zhang, Mingqian Huang, Junqing Chen, Quan Zhou, Yunfei Zhu, Rong Hua, Jianyu Tian, Xin Wang, Zuomin Zhou, Jie Hao, Jinjin Yu, Dong Hua, Jiayin Liu, Xuejiang Guo ✉, Qi Zhou ✉ & Jiahao Sha ✉

The gonads of aborted **12 - to 19- week male fetuses**

- The culture medium:
  - αMEM + 10% KSR
  - BMP 4/7 (20 ng/mL)
  - SCF (20 ng/mL)
  - bFGF (20 ng/mL)
  - EGF (20 ng/mL)
  - GDNF (20 ng/mL)
  - Activin A (100 ng/mL)
  - **Testosterone (10 mM)**
  - FSH (200 ng/mL)
  - BPE (50 mg/mL)
  - With or without RA (10^{-6} M)



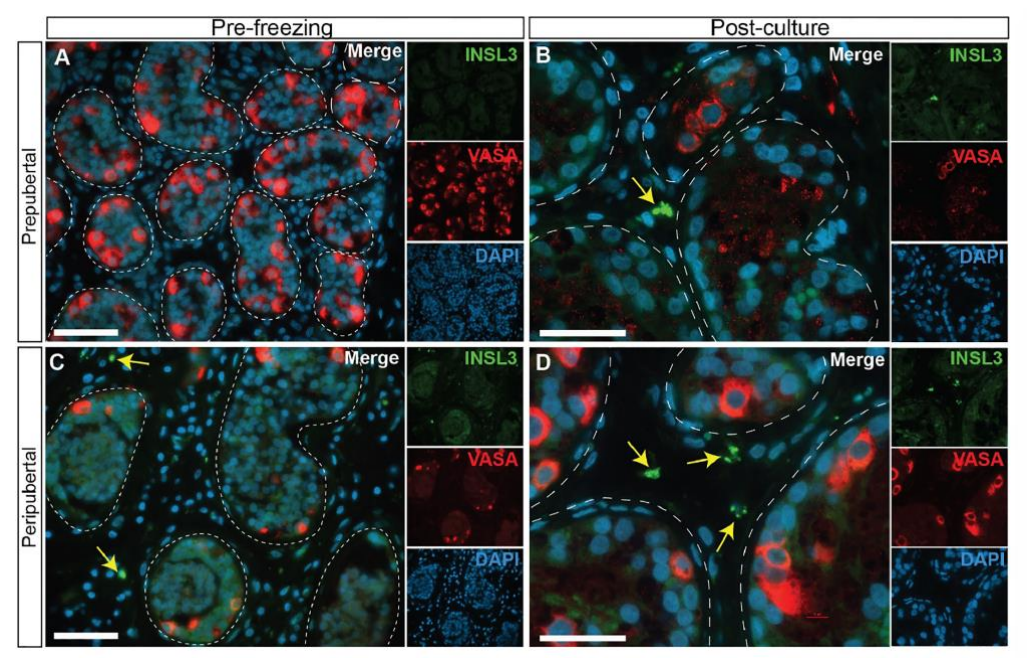


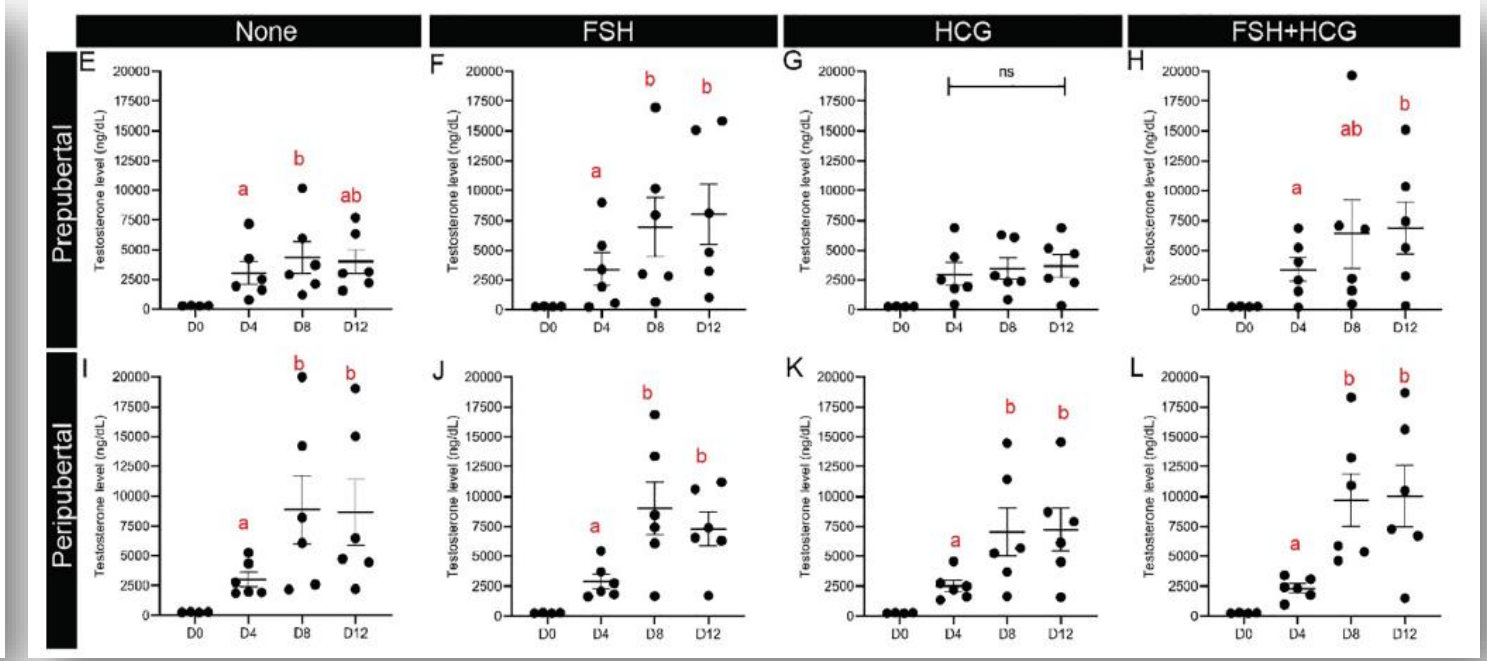
Yuan et al., 2020

IVM: how to move forward?

- Study the impact of individual components of culture media

INSL3-VASA





HCG alone: no increase in testosterone secretion

Younes et al., 2023

Conclusion

- IVM of human prepubertal tissue led to haploid germ cells in very limited amounts precluding any further characterization (genetic and epigenetic)

- A systematic approach to identify culture requirements will not be possible without collaborative efforts due to the scarcity of ITT for research purposes

- More research is urgently awaited to find solutions for patients with cancer cells contaminated tissue

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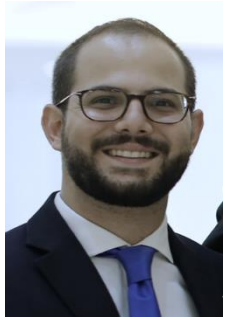

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Hanne Vlieghe
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Merci de votre attention

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REPR Research Pole in Reproductive Physiopathology

SALUS SANGUINIS
L'espoir coule dans nos veines