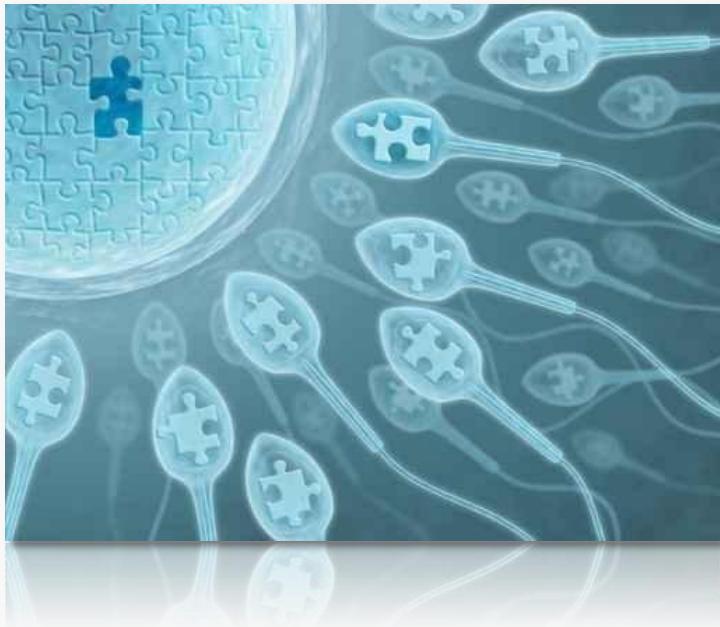




*12^{èmes} journées sur l'Assistance Médicale à la Procréation de
l'Hôpital Américain de Paris - 24-25 novembre 2011*



La protéomique de l'éjaculat

Projet Fertichip™

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Instituts thématiques
Inserm
Institut national
de la santé et de la recherche médicale

 **irset**
Institut de recherche sur la santé
l'environnement et le travail
Inserm U1085

UNIVERSITÉ DE
RENNES 1

 **Biogenouest**
Riomédiobiotech

La Protéomique

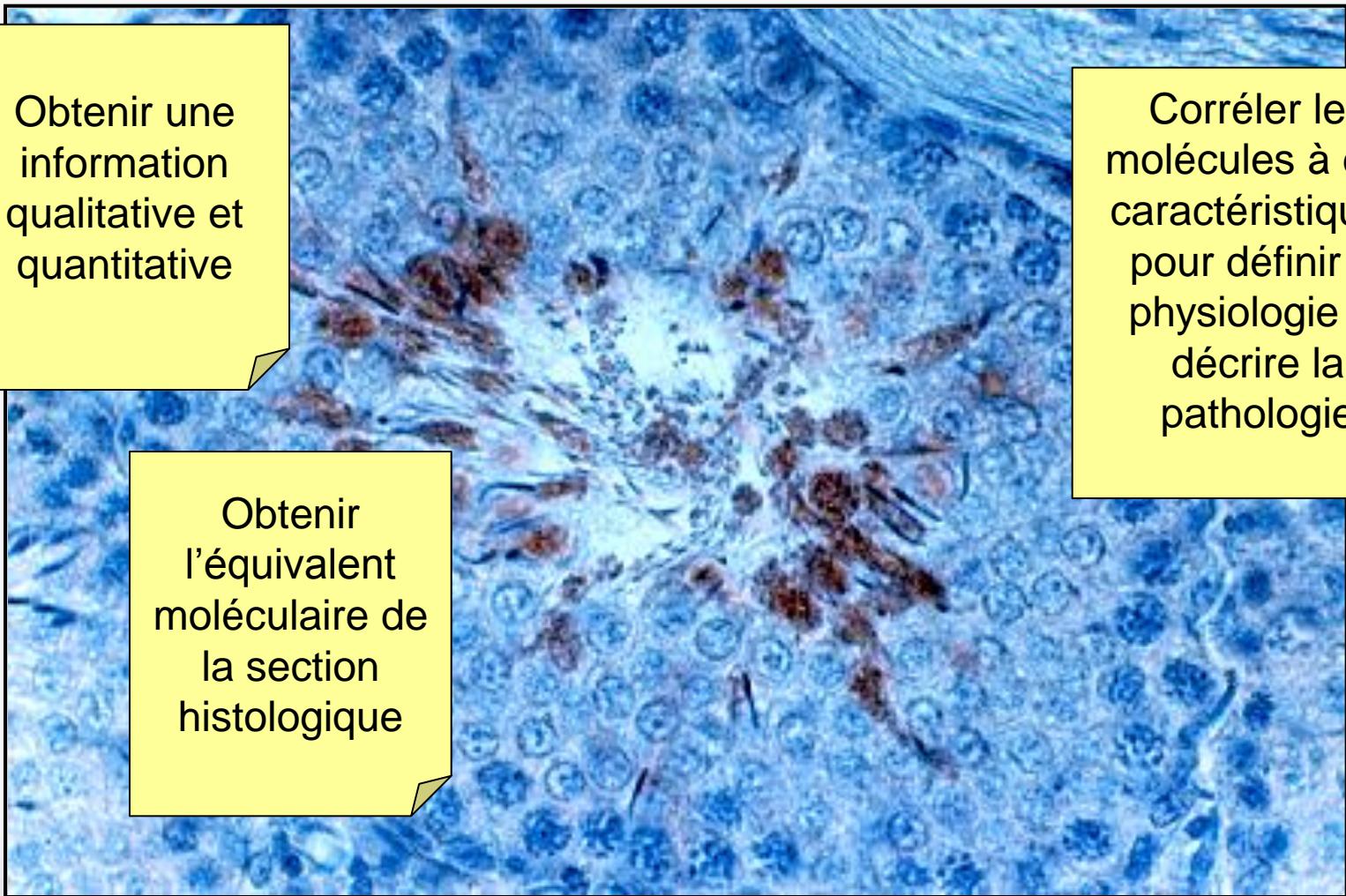
Etude des protéines à grande échelle par des méthodes de biochimie et de biologie structurale, en relation avec les données du génome

Trois domaines:

- **Cartographie et identification des protéines**
- **Analyse des interactions / recherche de partenaires**
- **Structure / Fonctions**



Pourquoi la protéomique ?



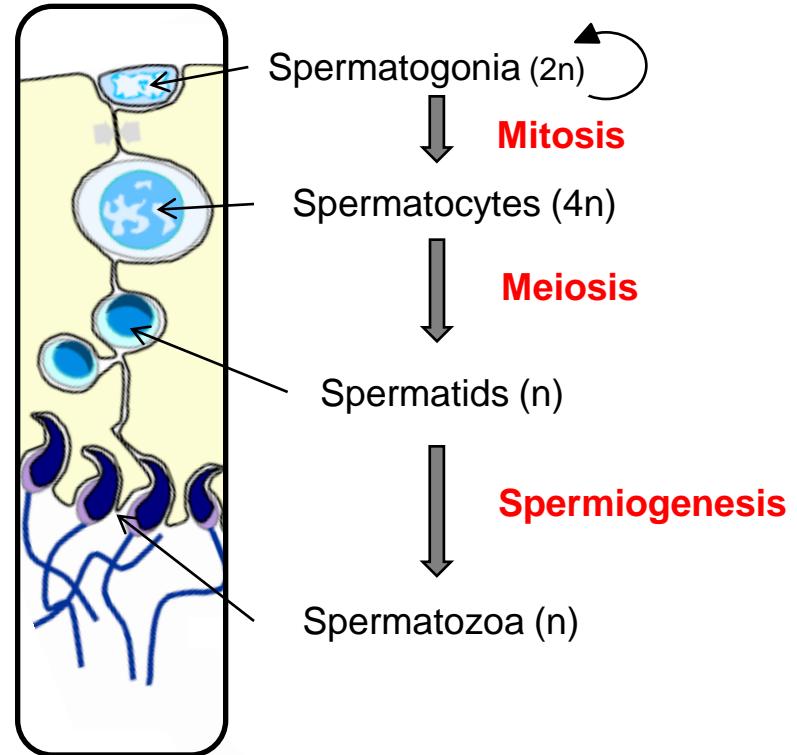
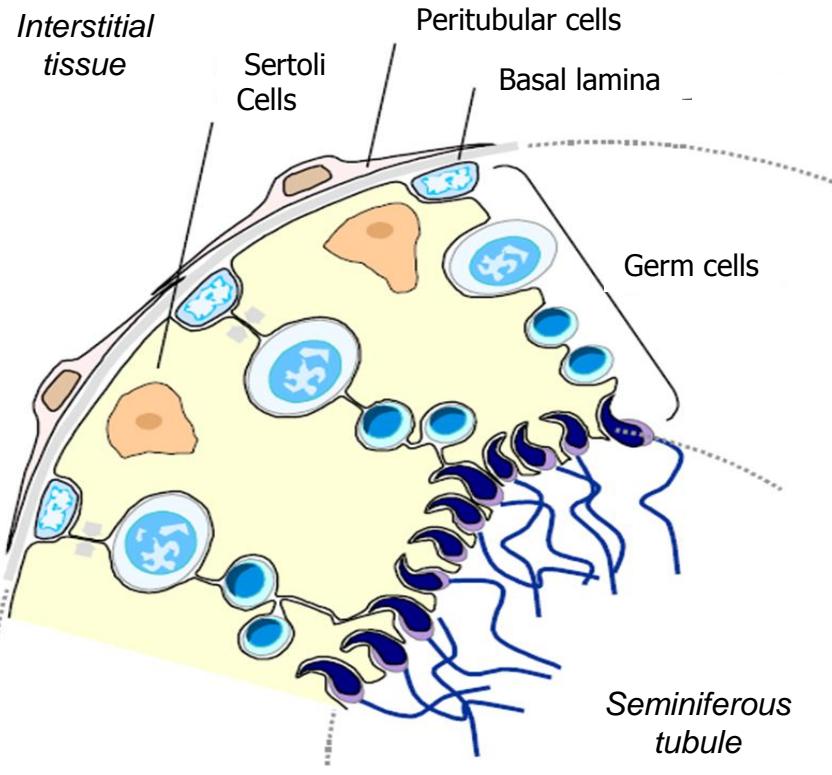
Obtenir une information qualitative et quantitative

Corréler les molécules à des caractéristiques pour définir la physiologie et décrire la pathologie

Obtenir l'équivalent moléculaire de la section histologique

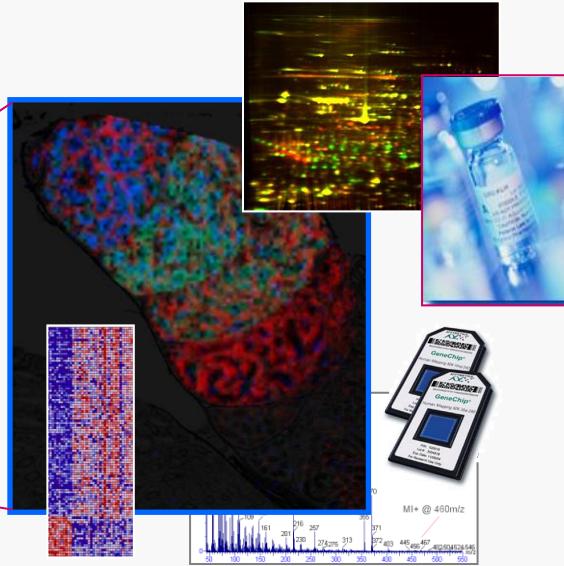
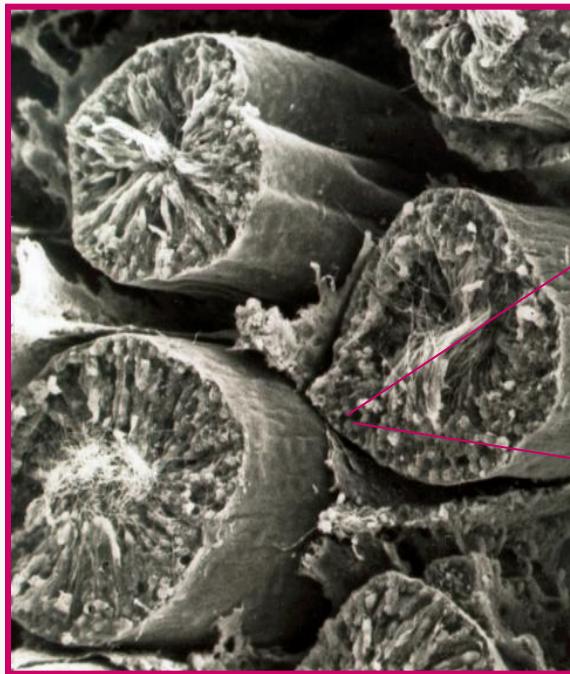
Approche permettant l'évaluation holistique de l'expression des gènes dans une cellule ou un tissu

Spermatogenesis in mammals



Our objective:

Decipher the intercellular crosstalks that drive germ cell maturation



The Testis Proteome Project

- Establish the repertoire of testicular proteins
- Identify key proteins in spermatogenesis
- Explore testicular pathologies (*e.g., sterilities, CIS*)

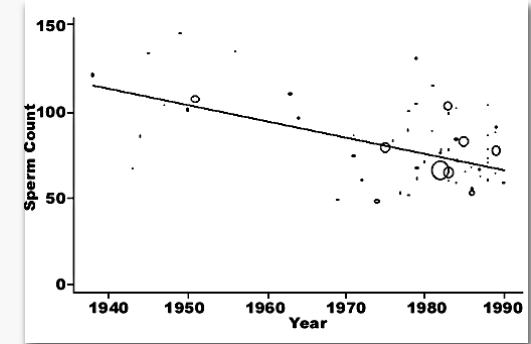


Protein markers for clinical applications

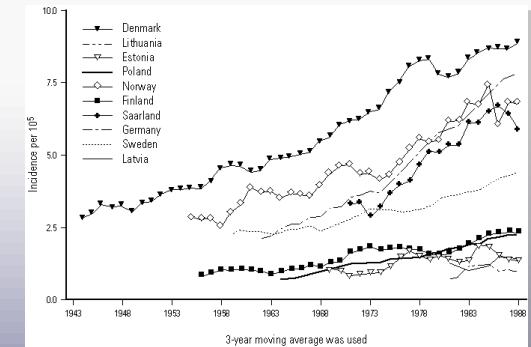


Status of male fertility

- 15% of couples have fertility problems in developed countries
- In a couple, fertility problems are shared equally
- Unknown etiology for 1/3 of cases
- No cure for most of hypofertilities



- Sperm decline in developed countries



- Increasing numbers of testicular cancers

Clinical care of male infertility

- **Male infertility:**

- **Obstructive Azoospermia = secretion cause**
- **Non Obstructive Azoospermia = excretion causes**

- **Obstructive azoospermia rather easy to diagnose**

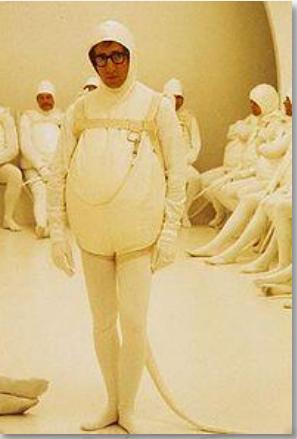
- **In vitro fertilization program - NOA within couple**

1. **Sperm extraction from testicular biopsies (TESE)**
2. **In vitro fertilization with intracytoplasmic sperm injection (ICSI)**

Or: Third party reproduction

Or: Alternative family building

How to predict a positive biopsy outcome?



The diagnosis of Non Obstructive Azoospermia

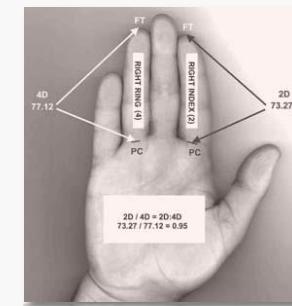
Is there a chance to retrieve live sperm cells from testicular biopsies?

- Spermogram; sperm biochemistry; plasma FSH, LH, prolactin...

- Physical examination parameters:



Testis size



2D:4D finger length ratio

- Assays:



Inhibin A and B, AMH



Indirect tests with very poor predictive power



The market of Assisted Reproductive Technologies

- ✓ 12 millions IVF cycles performed per year worldwide
- ✓ >10% involve male infertile patients

Source: World Health Organization

Number of biopsies performed per year:

France	Italy	Spain	U.K.	Benelux	Brasil	USA
6 000	4 500	8 000	5500	6 000	8500	35 000

**~ 60% of testicular
biopsies are negative**

**No consensus test available
to predict positive biopsy outcomes**
Testicular biopsy is mandatory



The human seminal plasma: a source for potential biomarkers

Produced by 4 major organs:

**Production of spermatozoa
and testicular fluid**



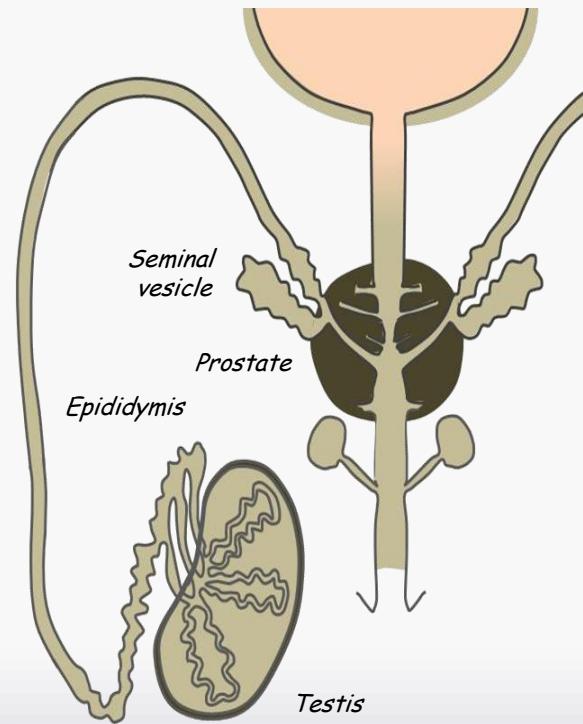
Transit and maturation of gametes



- Energy supplying (Proteins, Fructose...)
- Alkaline buffering



- Fluidization of ejaculate (Proteases...)
- Motility and final maturation of spermatozoa



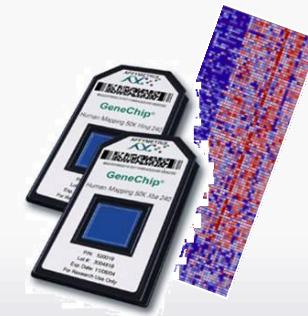
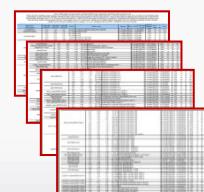
Experimental strategy

Differential analysis

A screenshot of a software interface for differential analysis, showing a grid of data with various columns and rows. A red box highlights a specific section of the table.

iTRAQ, Label-free, DIGE...

An alternative approach



AMEN: The Annotation, Mapping, Expression and Network suite of tools Chalmeil & Primig, BMC Bioinformatics, 2008

The Human seminal Plasma proteome



- > 2000 proteins identified to date
- Relatively poor mining information for male reproduction specialists
- Major effect of coagulation/liquefaction on proteome
- Several known seminal plasma proteins no yet found



Search for new discrete proteins
Identify organ/function markers

Comparison of human seminal plasma proteomic studies

Studies to be integrated:

- *Utleg et al., 2003*

=> **136** proteins

- *Fung et al., 2004*

=> **46** proteins

- *Pilch & Mann, 2006*

=> **916** proteins

- *Thimon et al., 2008*

=> **148** proteins

- *Drake et al., 2009*

=> **34** proteins

- *Poliakov et al., 2009*

=> **440** proteins

- *Wang et al., 2009*

=> **626** proteins

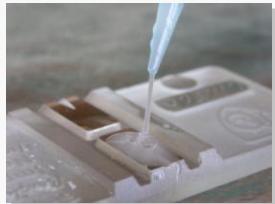
- *Batruch et al., 2011*

=> **2022** proteins

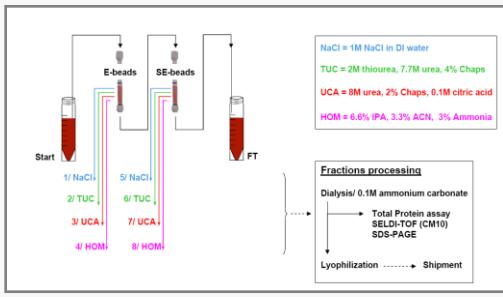
Identification lists

Protein	Accession	Peptides	Score	Confidence	Method	Sample	Replicate	Run	Time	Chromatogram
Protein 1	Q00000	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom1
Protein 2	Q00001	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom2
Protein 3	Q00002	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom3
Protein 4	Q00003	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom4
Protein 5	Q00004	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom5
Protein 6	Q00005	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom6
Protein 7	Q00006	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom7
Protein 8	Q00007	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom8
Protein 9	Q00008	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom9
Protein 10	Q00009	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom10
Protein 11	Q00010	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom11
Protein 12	Q00011	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom12
Protein 13	Q00012	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom13
Protein 14	Q00013	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom14
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Protein 16	Q00015	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom16
Protein 17	Q00016	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom17
Protein 18	Q00017	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom18
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Protein 22	Q00021	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom22
Protein 23	Q00022	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom23
Protein 24	Q00023	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom24
Protein 25	Q00024	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom25
Protein 26	Q00025	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom26
Protein 27	Q00026	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom27
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Protein 29	Q00028	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom29
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Protein 31	Q00030	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom31
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Protein 33	Q00032	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom33
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Protein 39	Q00038	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom39
Protein 40	Q00039	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom40
Protein 41	Q00040	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom41
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Protein 53	Q00052	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom53
Protein 54	Q00053	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom54
Protein 55	Q00054	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom55
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Protein 79	Q00078	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom79
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Protein 86	Q00085	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom86
Protein 87	Q00086	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom87
Protein 88	Q00087	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom88
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Protein 91	Q00090	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom91
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Protein 94	Q00093	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom94
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Protein 97	Q00096	123	45.6	High	LC-MS/MS	S1	R1	U1	12:00	Chrom97
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Experimental strategy

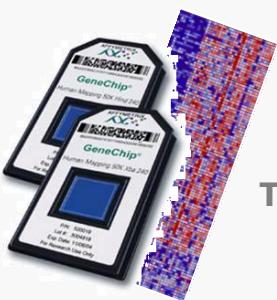


Human
seminal plasma



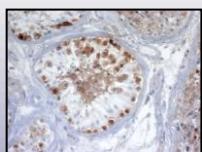
Proteominer™ fractionation

8 Fractions
+ Start
+ Flow Through

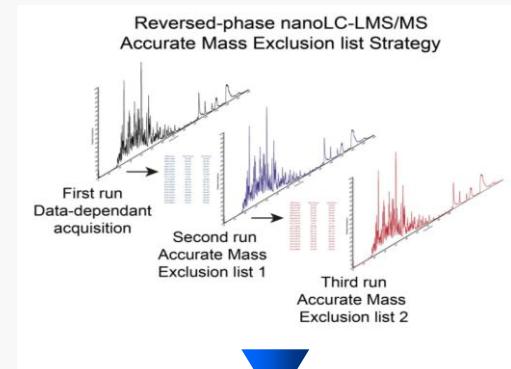


Mining with
Transcriptome datasets

Candidate marker selection



on normal and pathological
seminal plasma

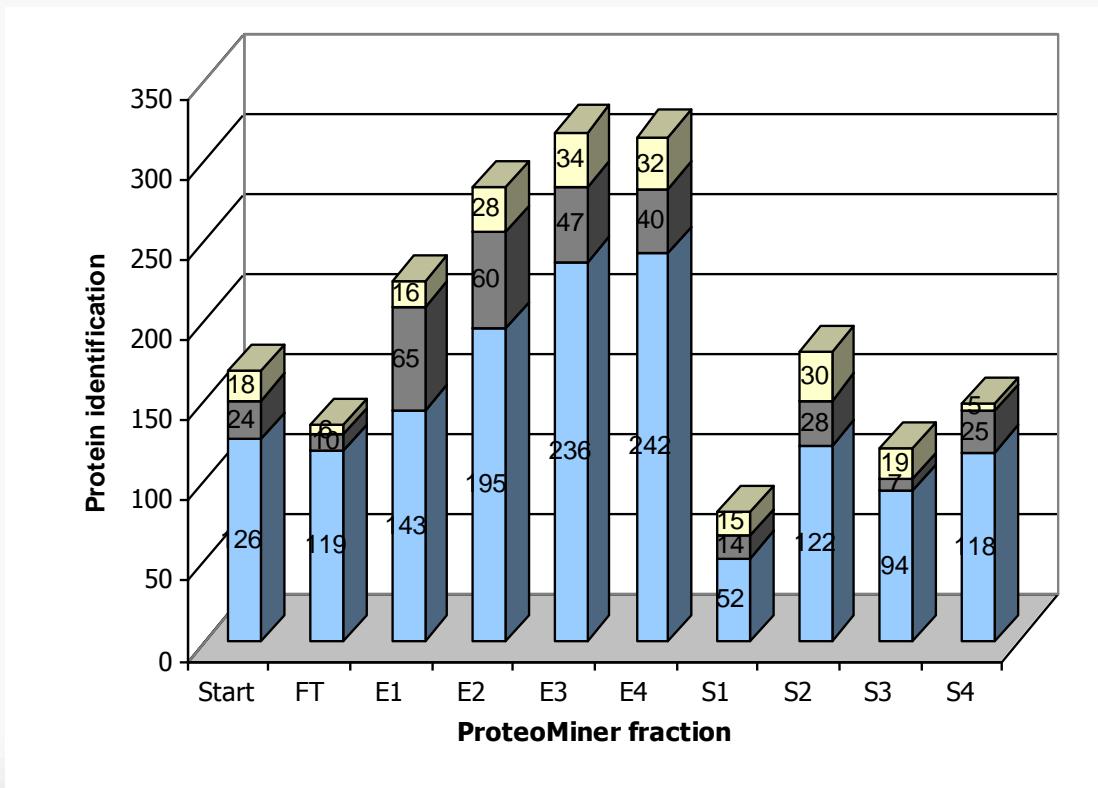


Combined
Dataset



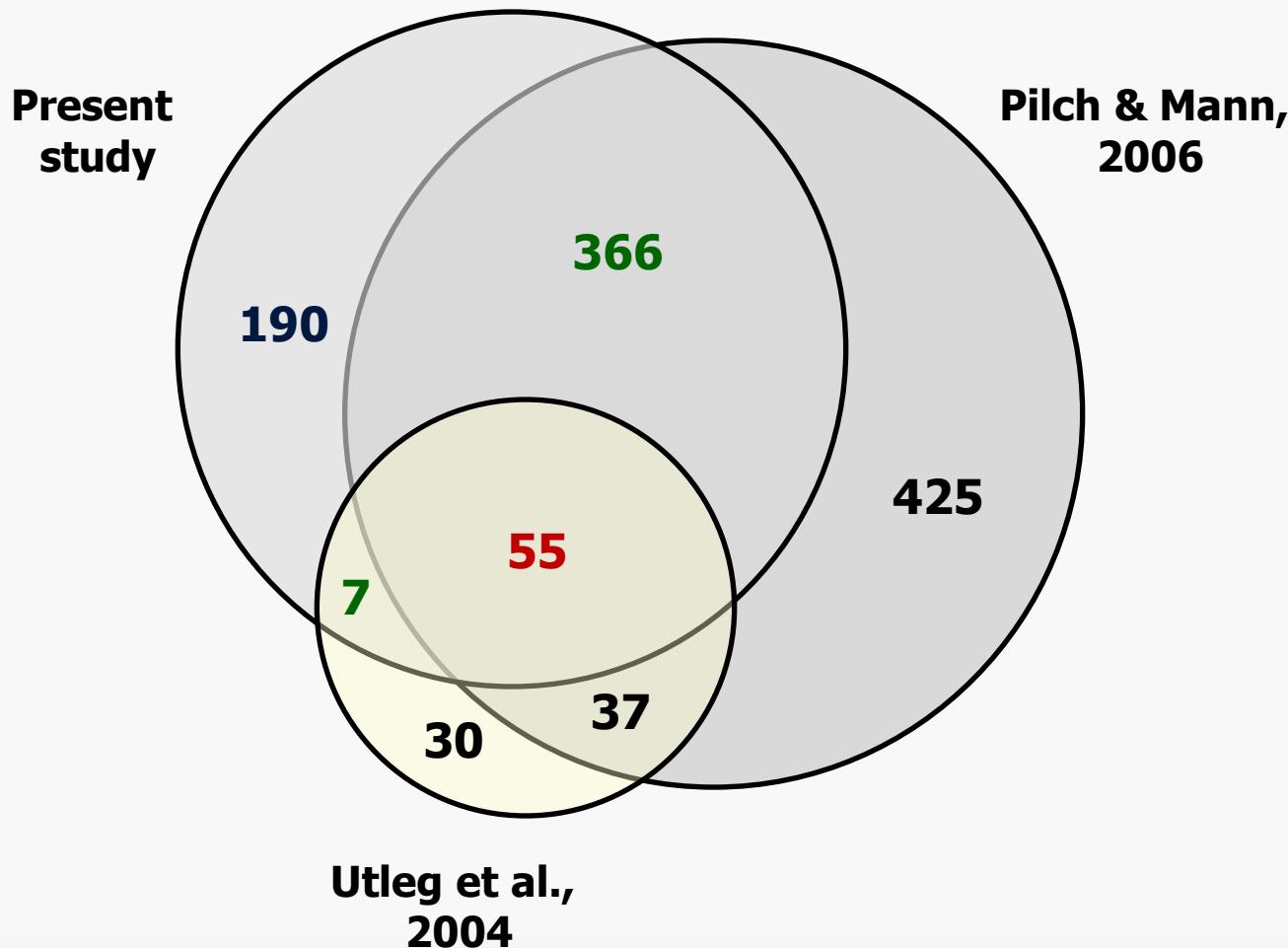
Bibliomics

Number of identified proteins from multiple injections of each fraction and dynamic exclusion of previously identified peptides



~20% more proteins in each fraction

Comparison of 3 proteomic studies on the human seminal plasma



Incomplete covering of the three datasets

Relevant approach in completing the repertoire of proteins

Comparison of human seminal plasma proteomic studies

Mapping of protein identifiers

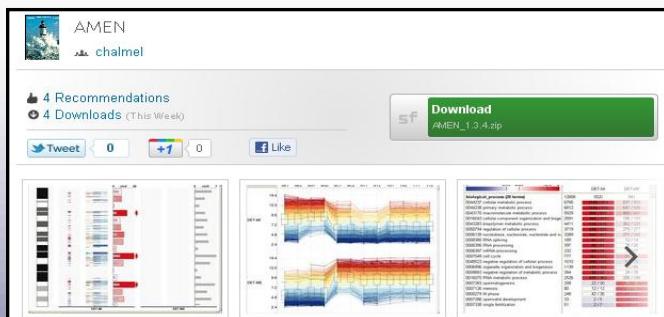
Identification lists



Uniprot Acc Numbers
Uniprot Entry Name
GI numbers
IPI Identifiers

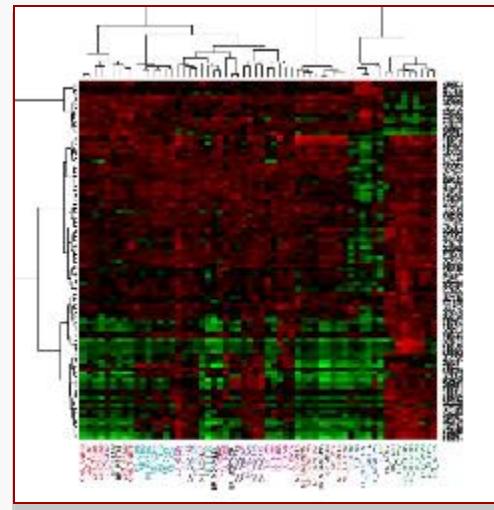
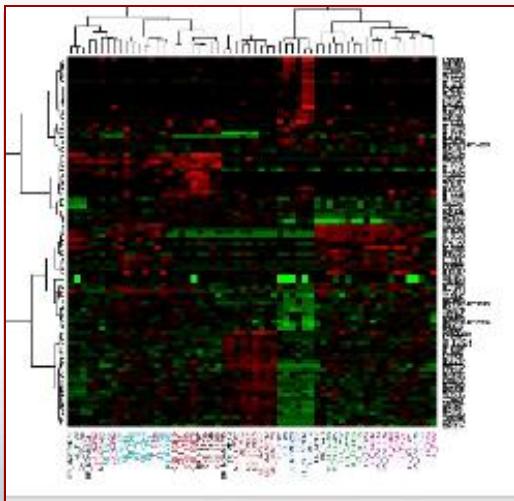
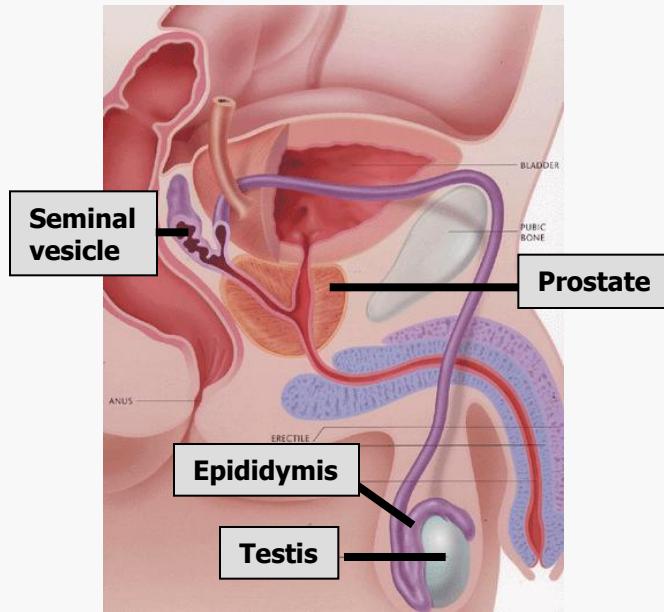
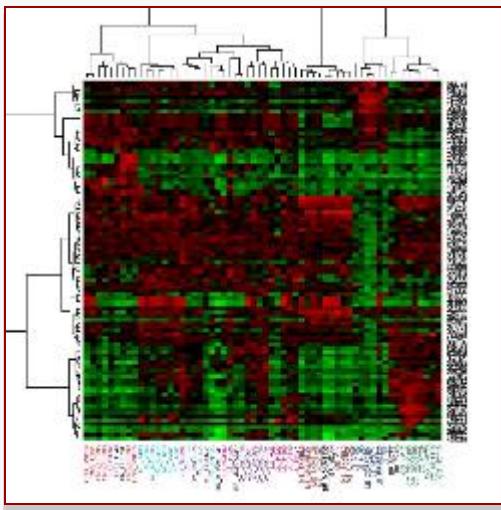
Uniprot Acc Numbers
EntrezGene

GeneOntology
Probeset

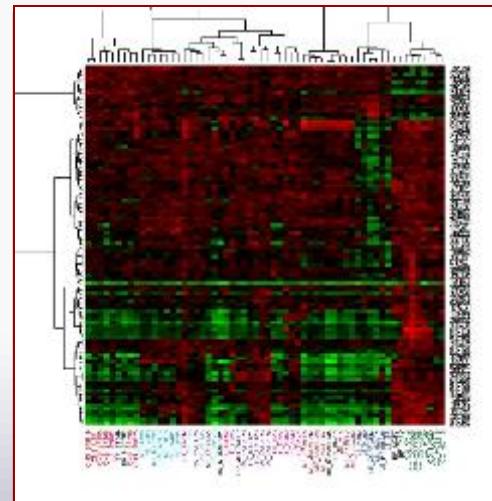


	Original IDs	Uniprot IDs	Entrez Gene IDs	Probe sets
Present study	668	668	676	1462
Pilch & Mann (2006)	916	875	881	1998
Utleg et al. (2003)	136	121	132	347
Fung et al. (2004)	46	48	45	99
Wang et al. (2009)	626	511	450	1024
Drake et al. (2009)	34	35	36	96
Poliakov et al. (2009)	440	443	446	949
Thimon et al. (2008)	148	146	127	298
Batruch et al. (2011)	2022	1970	2083	4771
Overall	-	2567	2331	5341

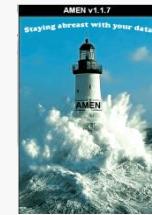
Mining protein identification list with transcriptomics datasets



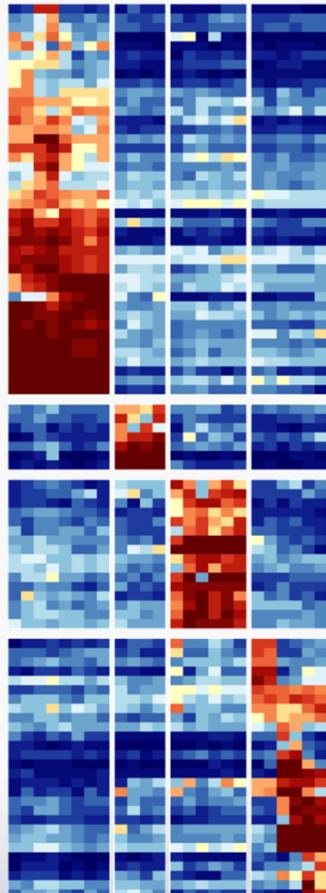
**Organs involved in
seminal plasma
production**



Mining protein identification list with transcriptomics datasets



Chamel & Primig,
BMC Bioinformatics 2008

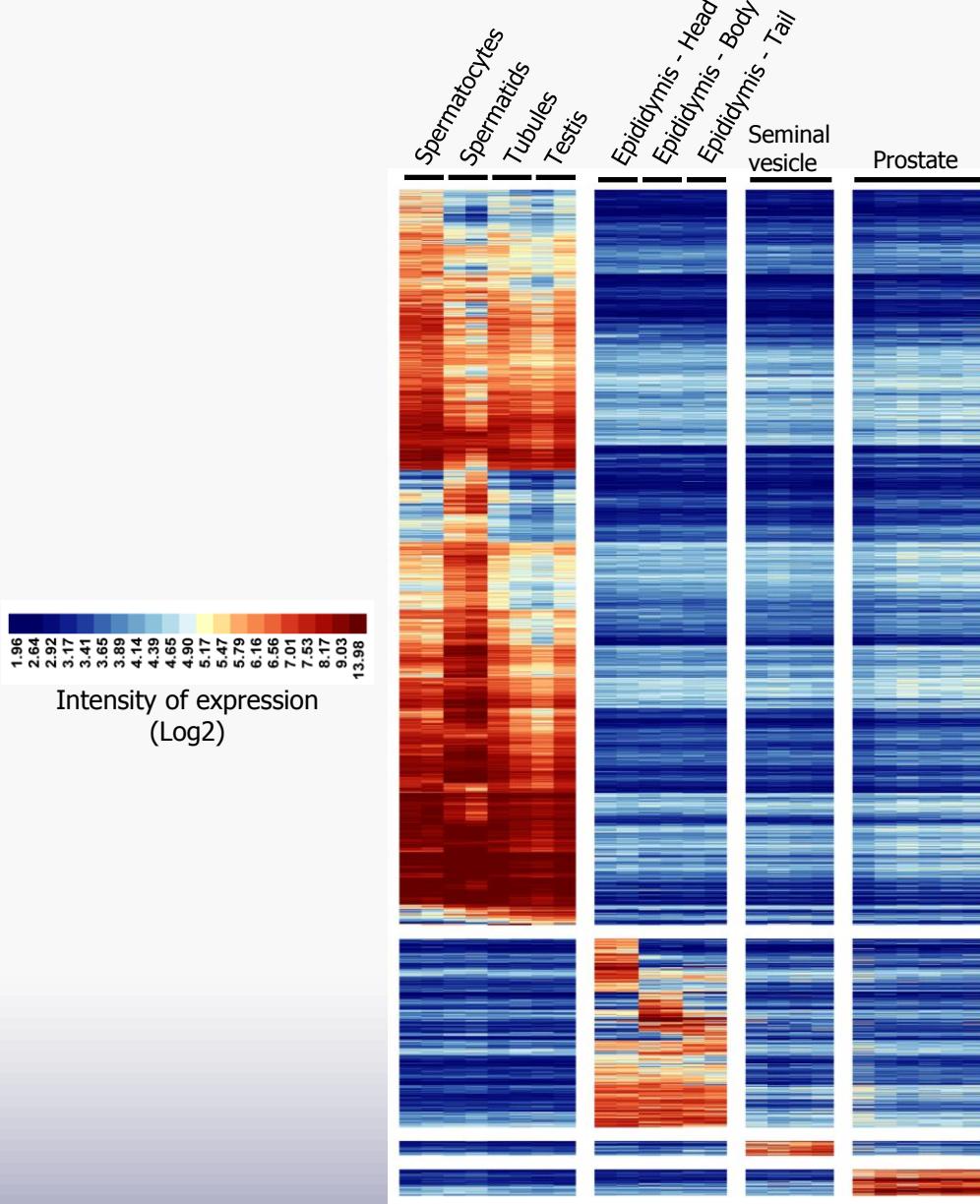


How to identify organ functional markers in the seminal plasma ?

Selection strategy

- 1. Identify overexpressed genes in organ datasets**
- 2. Verify organ-specificity of candidate genes**
- 3. Search for corresponding proteins into the protein list**

Organ-specific gene expression along the human male reproductive tract

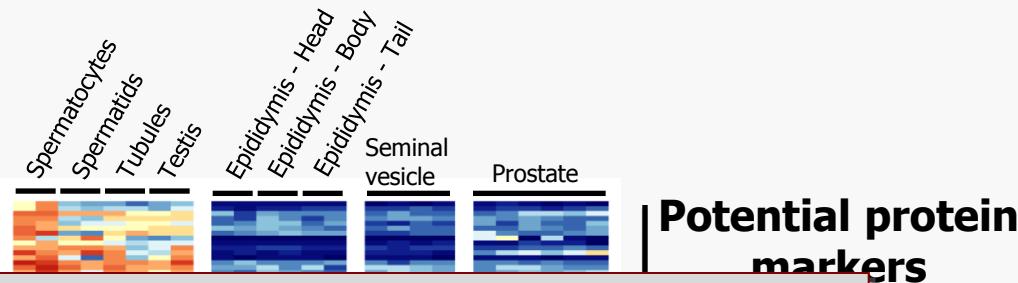


- **Detection Threshold = Mediane**
- **FC > 2**
- **Limma FDR <1%**

1942 genes
(2669 probes)

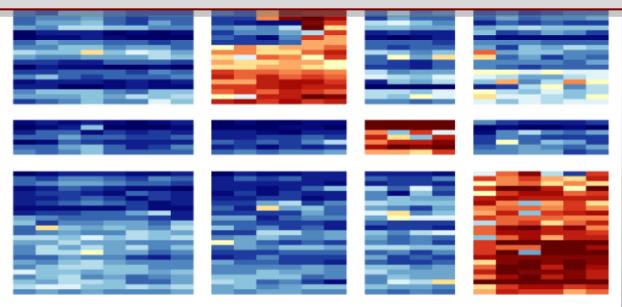
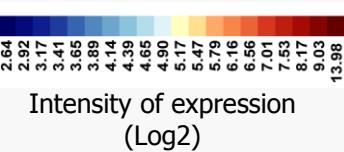
391 genes
(684 probes)
35 genes (53 probes)
53 genes (95 probes)

Tissue-specific protein expression along the human male reproductive tract



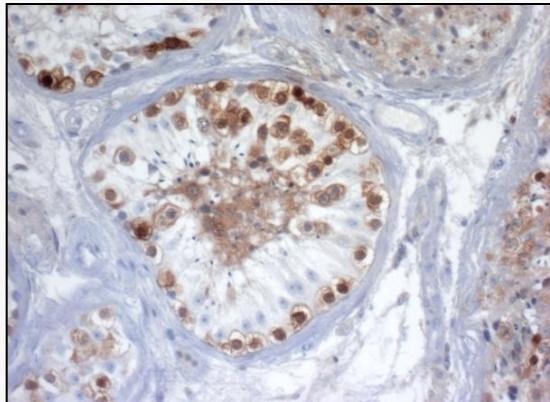
Verify the germ cell origin
of the proposed
candidates

?



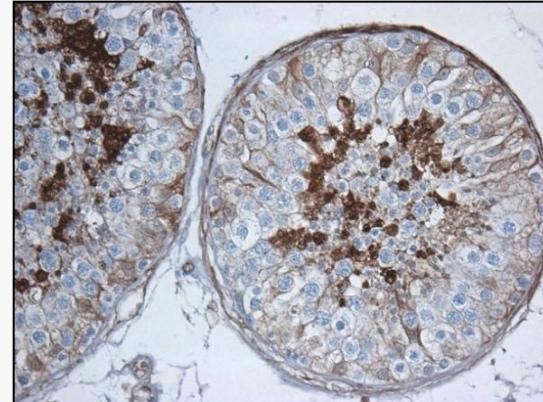
7
19

Validation of predicted markers of the urogenital tract



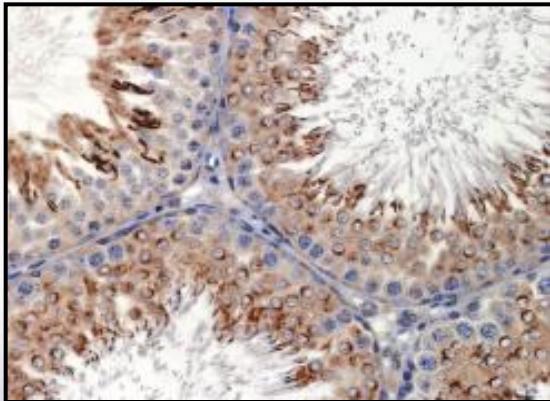
TKTL1

Secreted by spermatids



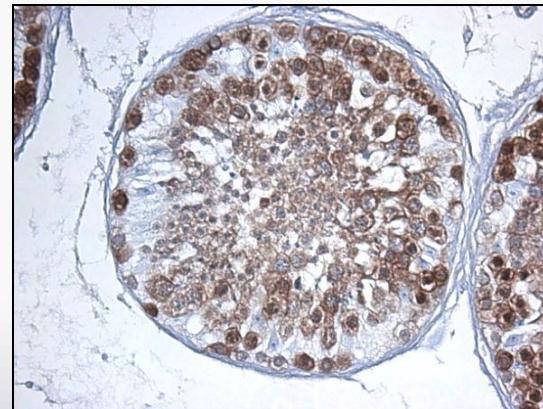
PGK2

Meiotic and post-meiotic



PGAMB

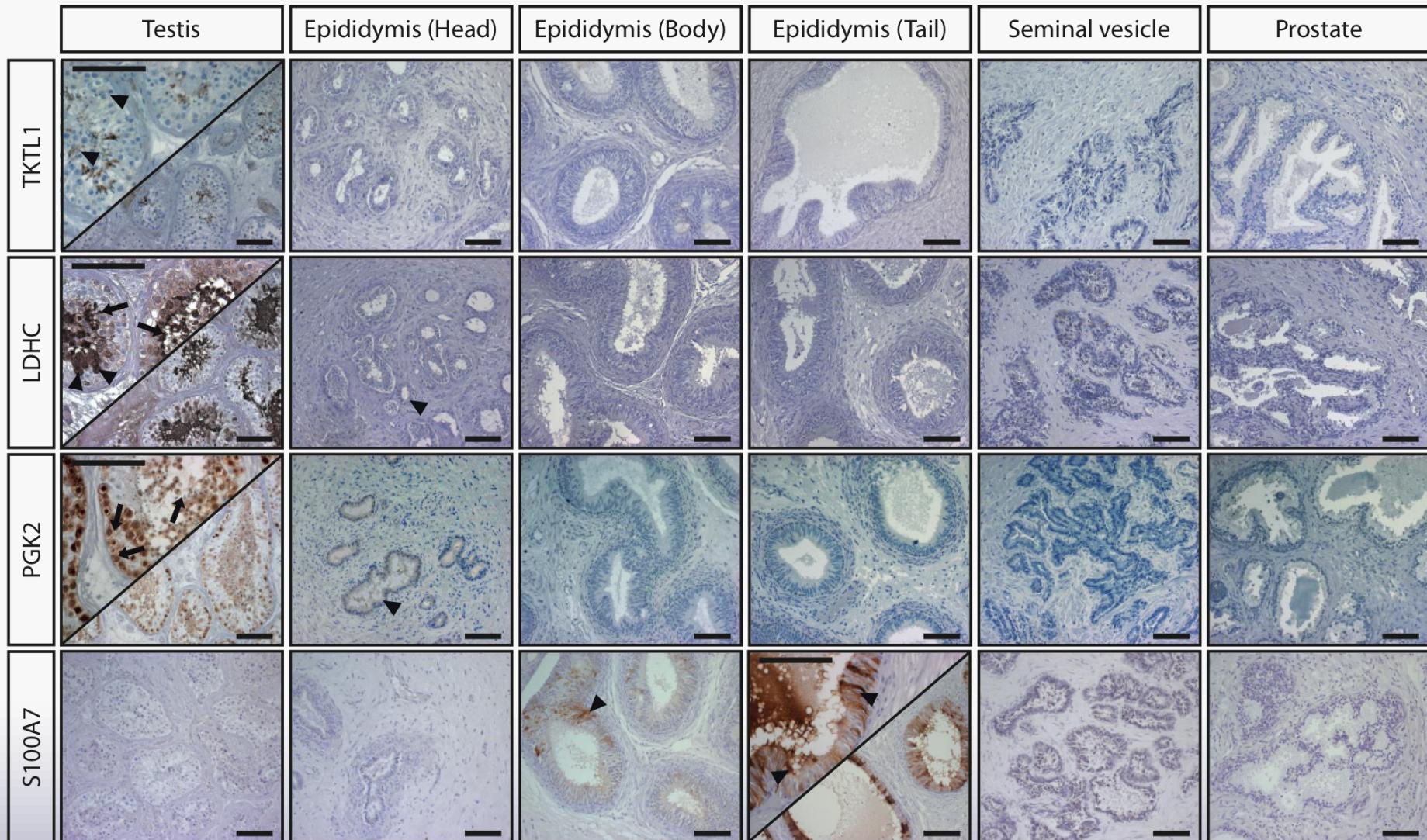
Meiotic and post-meiotic



LDHC

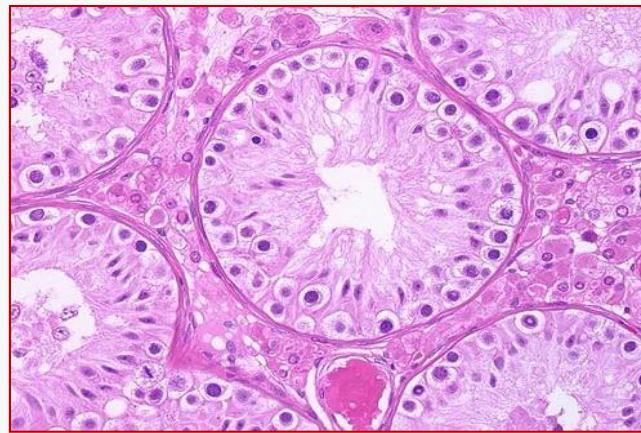
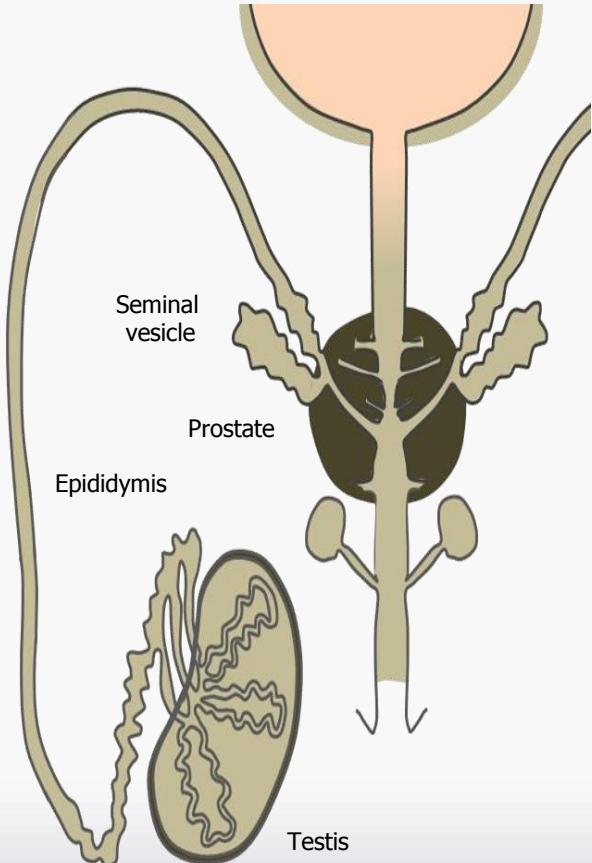
Meiotic and post-meiotic

Validation of germ cell line biomarkers

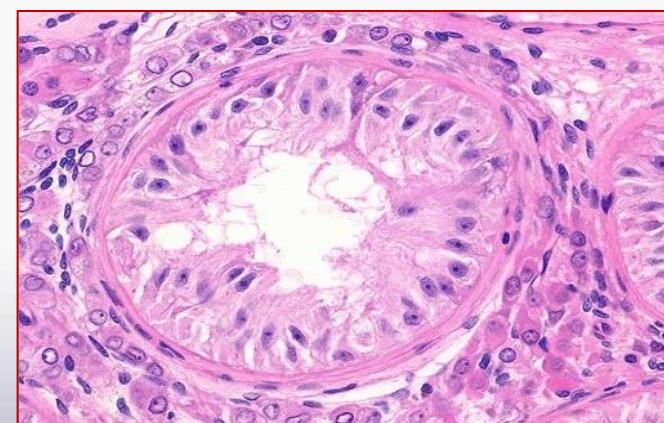


Most frequent Pathological situations

Non Obstructive Azoospermia



Normal spermatogenesis

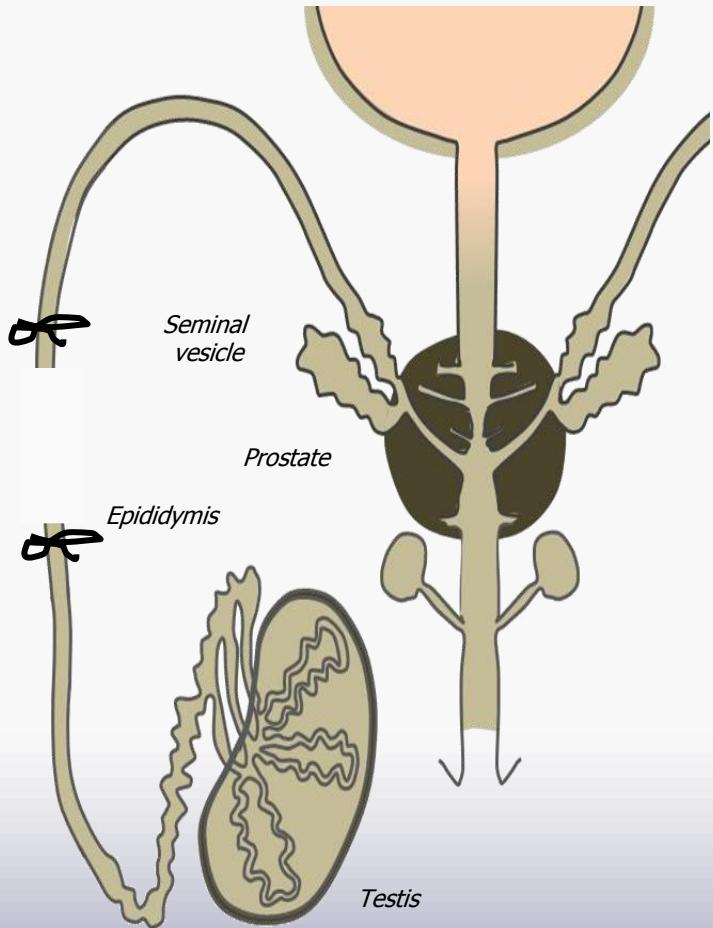
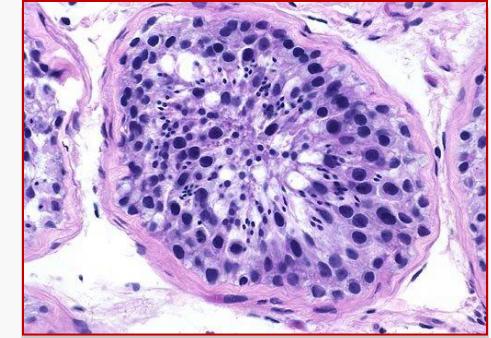


Maturation arrest

Sertoli-cell-only syndrome
(germ cell aplasia)

Most frequent Pathological situations

Obstructive Azoospermia



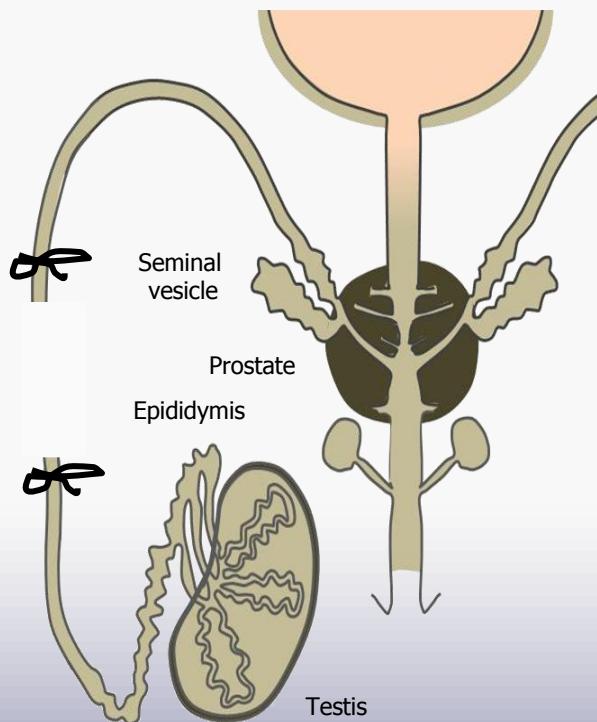
Validation of germ cell line biomarkers

Is it possible to discriminate each type of seminal plasma by assessing the presence or absence of several germ cell-specific biomarkers using ELISA assays?

Example 1:

Germ cell-specific TKTL1

protein secreted by spermatids



1-4: normal
5: unilateral obstruction
6-7: "CBAVD" ($\Delta f508$)
8-9: maturation arrest

10 11 12 13 14 15 16 17 18



10 -13: Normal (set #2)
14-16: Post-vasectomized
17-18: Sertoli-cell-only syndrome

Validation of germ cell line biomarkers

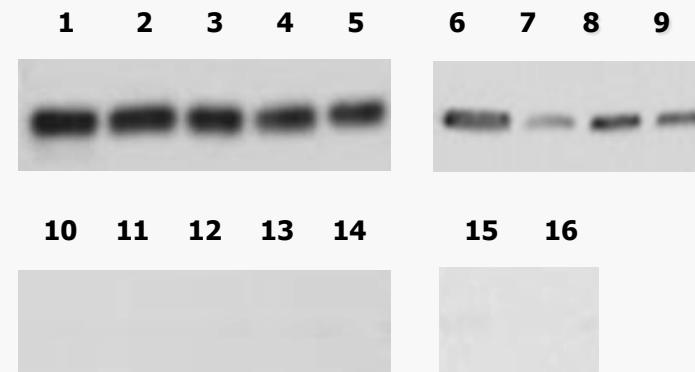
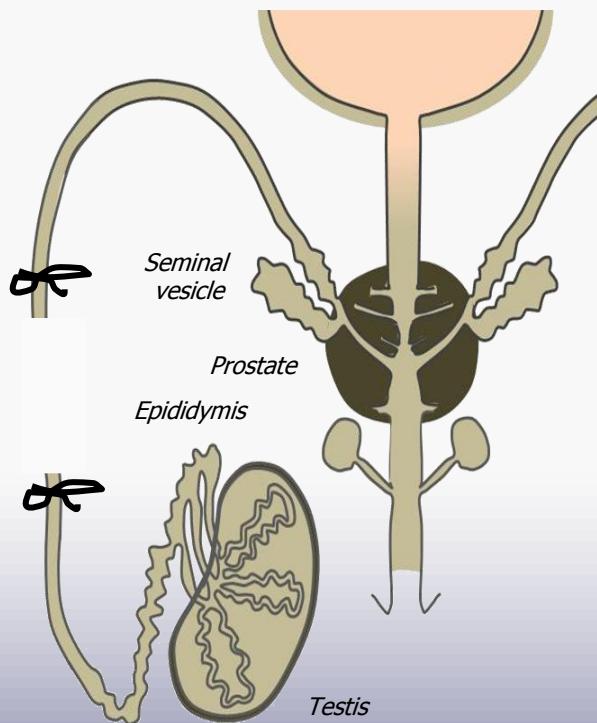
A blind retrospective study

Is it possible to predict the outcome of testicular biopsies?

Example 2:

Germ cell-specific PGK2

Meiotic & post-meiotic



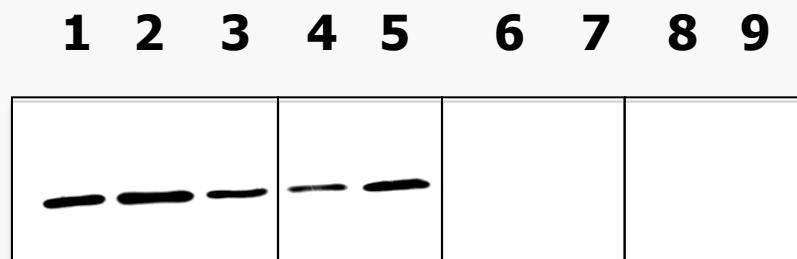
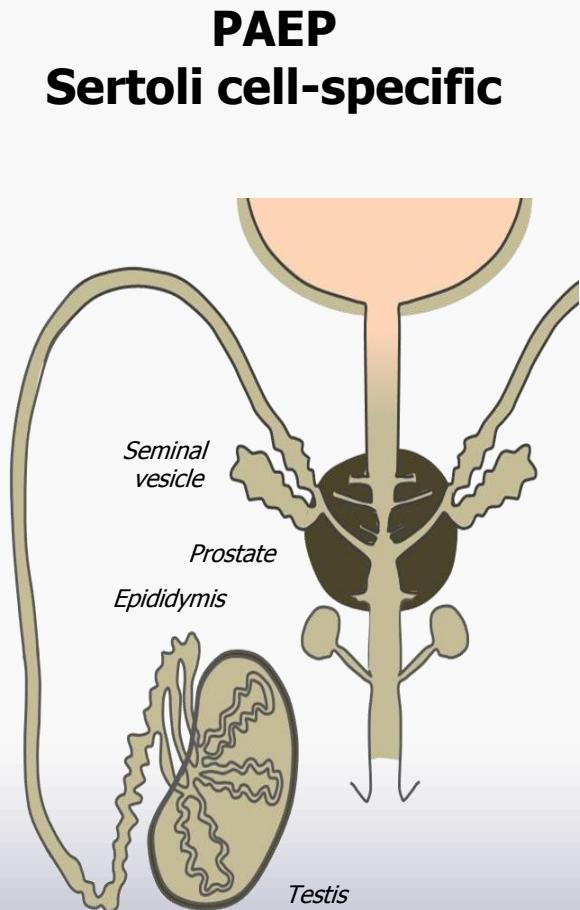
1-9: NOA with positive biopsies

10-14: NOA with negative biopsies

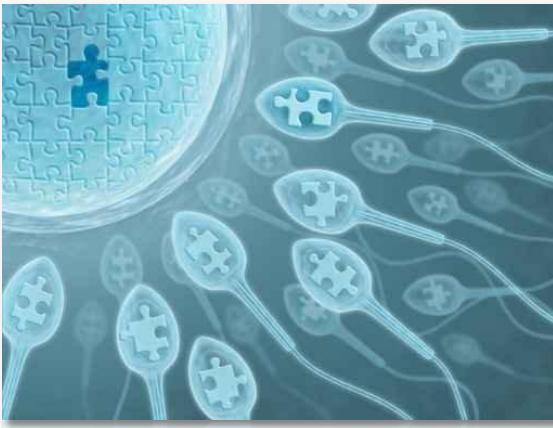
15,16: Post-vasectomy (used as control)

Additional validation of testis biomarkers

Example 3:



- 1- 3: normal**
- 4-5: Sertoli cell-only syndrome**
- 6-7: "CBAVD" ($\Delta f508$)**
- 8-10: Post-vasectomy**



The Fertichip™ concept

Patent application Inserm Transfert

Objective:

Assess the presence of mature germ cells in the testes of azoospermic patients

Method:

Detect the presence of germ cell-specific proteins in the seminal plasma using specific assays

Output:

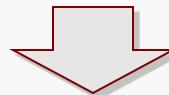
Provide a probability score that a testicular biopsy will be positive



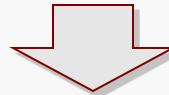
The Fertichip™ project



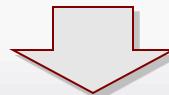
Selection, cloning of 91 germ cell specific genes and expression of recombinant proteins



Production and validation of monoclonal antibodies against each candidate protein



Development of sensitive ELISAs

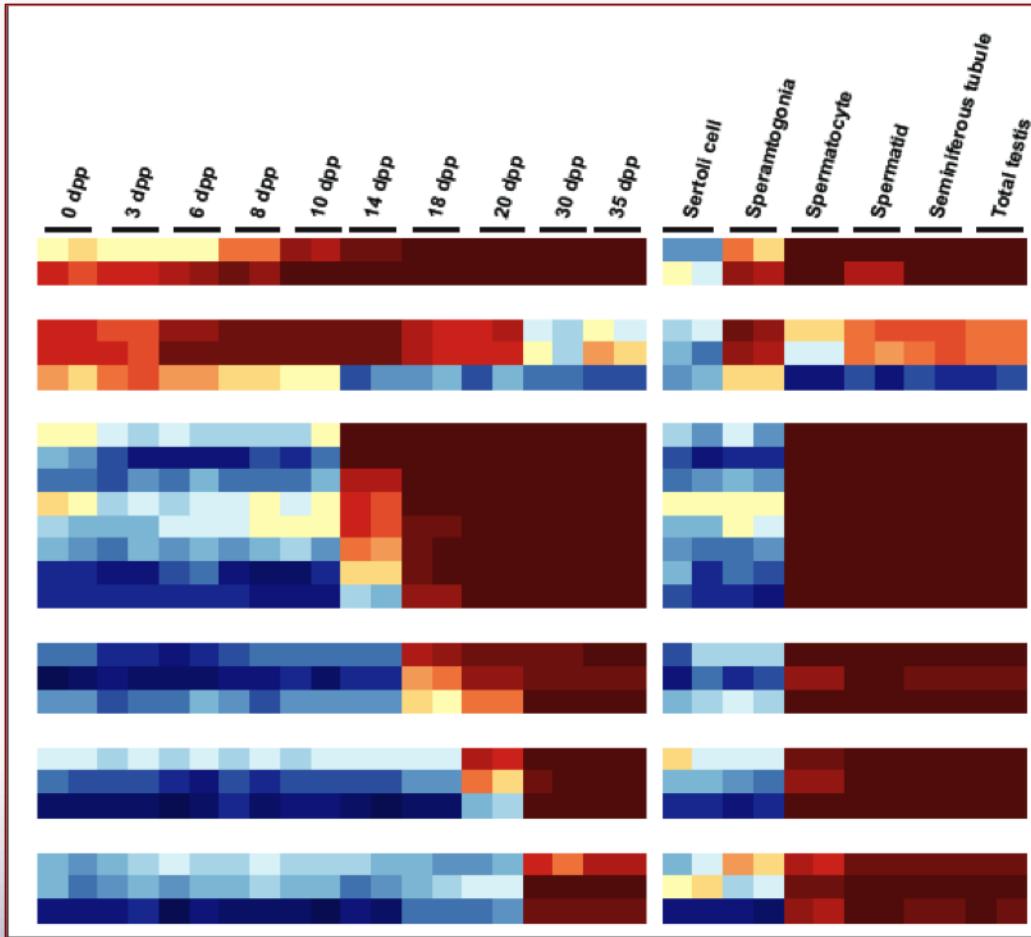


Development of a multiplex suspension bead assay (Luminex™)



Germ-cell specific markers in seminal plasma

Example of 22 top-priority candidates:



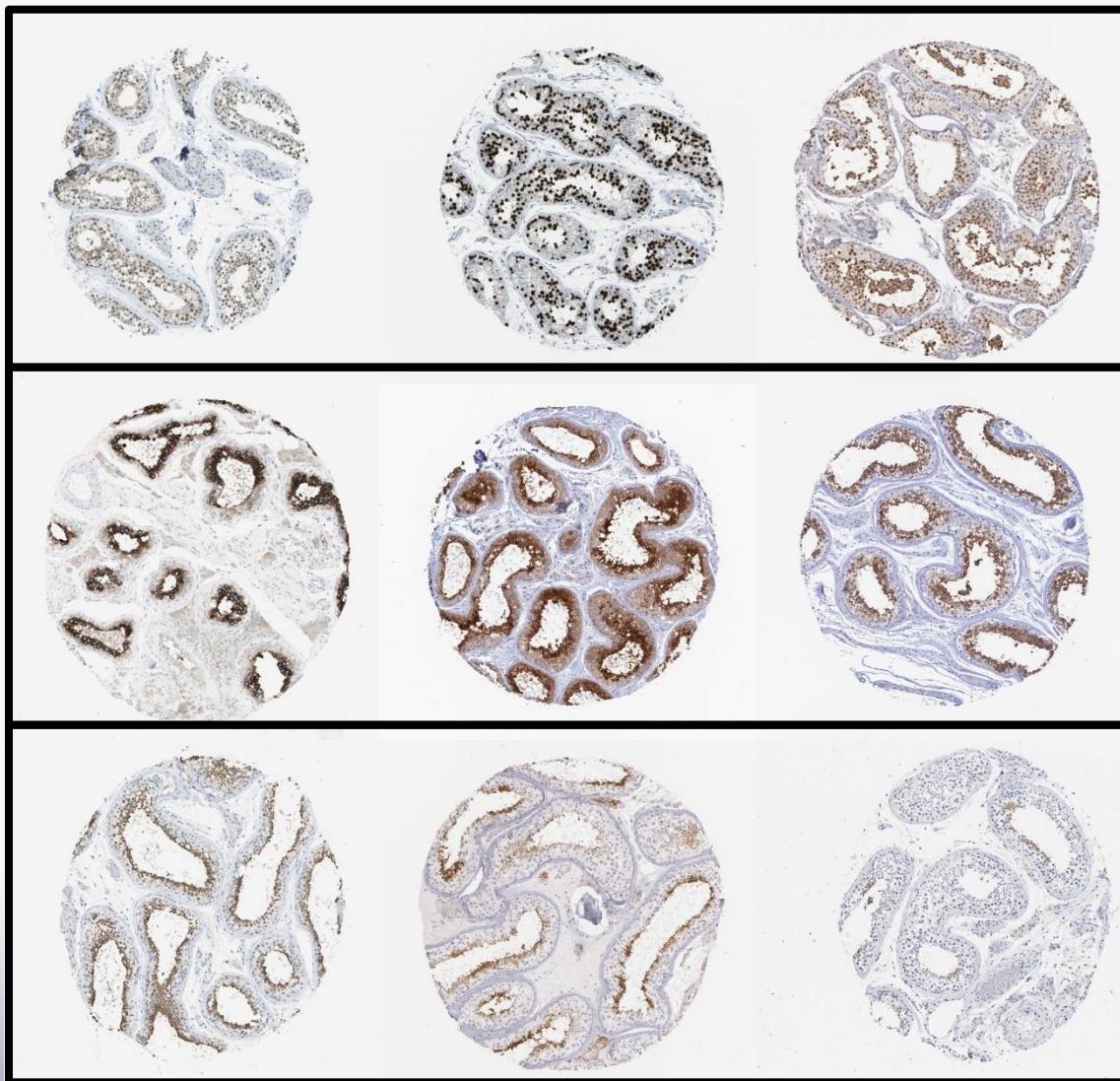
Total GC line

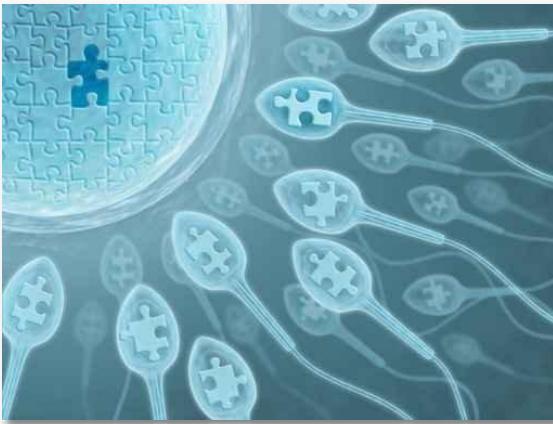
Spermatogonia

**Spermatocytes/spermatids
(meiosis)**

Spermatids

Ongoing: validation of germ-cell specific antibodies





The Fertichip™ assay

Validation step 1

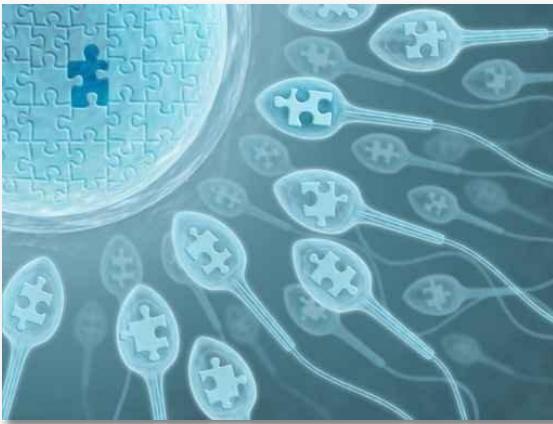
Is it possible to discriminate each type of seminal plasma by assessing the presence or absence of several germ cell-specific biomarkers using ELISA assays?

Cohorts: 400-1200 normal + pathological samples

Validation step 2

Is it possible to predict the outcome of testicular biopsies?

Cohorts: > 600 samples



The Fertichip™ assay

Validation steps

A complement to physical examination by the clinician to:

- Avoid unnecessary biopsies***
- Improve patient counselling**
- Diminish psychological costs**
- Reduce the financial cost of ART medicine**



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Catherine Celebi
Strasbourg

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Paris Cochin

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Rennes

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Brest

François Vialard
Florence Boitrelle
Poissy

Louis Bujan
Roger Mieusset
Toulouse



Spermatogenesis - Daniel DuVall 2000