A prostate perspective on male fertility and EASs: from toxicogenomics to phenotypic anchoring

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OUTLINE

- Introduction
  - Prostate as an overlooked toxicological target
  - PSA secretion in rodents
  - LNCaP as a model of human epithelium
  - Androgen Receptor (wt & mutated) and AR-mediated signalling

- The ReProTect project:
  - The ReproTect project: from toxicogenomics to phenotypic anchoring
  - Glufosinate ammonium

- The running projects:
  - The use of an in vitro alternative method to screen compounds with a pharmacological-like (anti-androgenic) activity
  - In vitro «bioavailability» of tested chemicals: nominal vs intracellular concentration
Male fertility depends from the activities of:
Testis, Epididymis, Seminal Vesicles and Prostate.

Prostate, an overlooked target in in vitro alternative methods, is essential for male fertility since it secretes the prostatic fluid (constituting ~ 30% of the whole ejaculate).
Indeed, sperm functional competence is depending on prostatic fluid that provides proteins (e.g. PSA and other kallikreins), trace elements (e.g. zinc) and other molecules (e.g. citrate) essential to sperm cell activation and capacitation.
PSA has a central role in semen liquefaction and, behind its established role as a prostate cancer/PCa biomarker, it might constitute a feasible toxicological biomarker due to its functional significance.

Lorenzetti et al., 2011, Annals Ist Sup Sanità, 47(4):429-44
One of the main features of the human prostate gland is not present in rodents, the classical animal models used in toxicology: indeed, the expression and secretion of the prostate-specific antigen (PSA or KLK3) is absent because the gene KLK3 emerged only after the separation of the primate and rodent lineages (Olsson AY & Lundwall A. BBRC 2002;299:305–11).

PSA secretion assay

Clinical/oncological biomarker

Functional biomarker

Toxicological biomarker

Phenotypic anchoring in toxicogenomics
✓ It is originated from a metastasis of prostatic adenocarcinoma (PCa)
✓ Tumorigenic in mice xenografts
  ▪ Horoszewicz et al., Cancer Res 1983; 43:1809–18.
  ▪ Webber et al. Prostate 1997; 30:58–64

✓ Androgen Sensitive
✓ It expresses a mutated androgen receptor (AR$^{T877A}$), the main one in human PCa
✓ A model of androgen-dependent PCa
✓ PSA (Prostate-Specific Antigen) is secreted and AR-modulated
✓ Under proper growth condition, it expresses only the sex steroid receptor AR and ER$\beta$ and no ER$\alpha$ as it occurs in physiological condition at the onset of PCa
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THE ANDROGEN RECEPTOR / AR: wild type and mutated

**Ligand Binding Domain (LBD):** PCa > 90% mutation in LBD; about 50% are AR$_{T877A}^\text{T}$
In PCa, AR- and ERα-mediated signalling cooperates not at the PCa onset but only later during PCa progression when more chemicals are activating them since: ERα gene expression is derepressed and the mutated AR\textsuperscript{T877A} has more ligands than usual.
In our experimental culture conditions, only ERβ and AR^{T877A} are expressed whereas ERα is NOT expressed. Thus allowing us to reduce the redundancy between AR and ERα signalling on common target genes.

Maranghi et al., 2007
Thematic Priority
Development of new \textit{in vitro} tests to replace animal experimentation

Project title
Development of a novel approach in hazard and risk assessment of reproductive toxicity by a combination and application of \textit{in vitro}, tissue and sensor technologies

WorkPackage 4
\textit{(WP4} coordinator: Alberto Mantovani\textit{)}
Strategies for \textit{in vitro} test batteries in reproductive/developmental toxicity: a toxicogenomic approach involving expression / regulation of androgen receptor genes

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ReProTect
The androgen receptor directly regulate development, maturation, functionality and homeostasis of the prostate gland and, in particular, of the prostate epithelium that secrete the prostatic fluid (1/3 in volume of the male ejaculate) an essential component to ensure male fertility.

Testicular Dysgenesis Syndrome (TDS): exposure *in utero* to environmental factors (anti-androgenic compounds) in Western Europe and USA nei paesi dell’Europa occidentale e negli USA, are responsible of male infertility and associated-diseases/malformations.

Adapted from Skakkebaek NE et al., 2001, Human Reproduction 16: 972–8
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FROM TOXICOGENOMICS TO PHENOTYPIC ANCHORING… (1)

cDNA microarray (toxicogenomic) unpublished data;

Lorenzetti et al., manuscript (1) in preparation
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FROM TOXICOGENOMICS TO PHENOTYPIC ANCHORING… (2)

Supplementary Figure 1 Ngan et al

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Ngan et al., Oncogene 2009
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FROM TOXICOGENOMICS TO PHENOTYPIC ANCHORING… (3)

PSA secretion unpublished data: time- and DHT dose-dependency;
PSA/KLK3 gene expression unpublished data;

Lorenzetti et al., manuscript (1) in preparation
Because PSA secretion is a:

- clinical biomarker in androgen-dependent prostate cancer
- functional & toxicological biomarker for human prostate epithelium
- direct target of AR-modulated signalling (its mRNA & protein expressions, too)
- linking gene expression (by qPCR or cDNA microarray) to a clinical and functional biomarker (PSA secretion itself) it allows the phenotypic anchoring.

Could we screen and prioritize environmental and dietary contaminants, having a role in prostate epithelium (in an in vitro model of an early stage of androgen-dependent PCa and of male fertility of ageing males), by using PSA secretion as a functional biomarker of their potential adverse/beneficial role?
Screening 10 chemicals in a double blind feasibility study:

- 2 confirmed as AR-interfering chemicals (vinclozolin and BPA)
- 1 newly identified as androgen-like chemical
Chemicals with specific effects on PSA secretion

Glufosinate ammonium / GA is a herbicide inhibiting selectively glutamine biosynthesis
Schulte-Hermann et al., 2006 Regul Toxicol Pharmacol.

Surprisingly, its effect on PSA secretion overlaps those one of the tested androgenic chemicals (DHT & MT)
Lorenzetti et al., 2010, Reprod.Toxicol.

DHT induces PSA secretion 5-12 fold
(in comparison to CTRL cells)
whereas GA induces PSA secretion up to 8-fold at 0.1 mg/ml
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GLUFOSINATE AMMONIUM (2)

- Glufosinate Ammonium (F2) is present in herbicide formulas specific for OGM “Liberty Link®”
- Known toxicological effect (in vivo): inhibition of embryo implantation
  ✓ Schulte-Hermann R et al., Regul Toxicol Pharmacol 2006; 44:S1–76.
- Glufosinate Ammonium (F2) does not bind the Androgen Receptor /AR wild-type!

Effect on PSA secretion dependent on AR^{T877A} (LNCaP cells)?

as bisphenol A / BPA (BPA does not bind the AR^{wt} but the AR^{T877A}


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GLUFOSINATE AMMONIUM (3)

GA dose-dependency of AR and PSA gene expression unpublished data;

_Lorenzetti et al., manuscript (2) in preparation_
FROM PHENOTYPIC ANCHORING TO AN INDEPENDENT SCREENING TOOL... (3)

OLD & NEW CHEMICALS (>40) UNDER INVESTIGATION ...

*Projects in progress:*
Italian Ministry of Health and Rovereto Town Council national projects

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<th>Drugs</th>
<th>Plant bioactives</th>
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<th>GMO-Herbicides</th>
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<th>Fungicides &amp; metabolites</th>
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<td>Di-n-butyl phthalate (DBP)</td>
<td>Glufosinate ammonium (GA)</td>
<td>Terbutylazin e (TBT)</td>
<td>Vinclozolin (VIN)</td>
<td>Vinclozolin M1</td>
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<td>Glyphosate</td>
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<td>Nitrofen</td>
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1. **In silico**
   - Molecular Docking

2. **In vitro**:
   - **2a** Cytotoxicity (MTS assay) test
   - **2b** Protein (free and total PSA) secretion assay
   - **2c** Gene expression: real time RT-PCR (qPCR) of the 48 human Nuclear Receptors (hNRs)
   - **2d** Chemical Biodistribution

3. **Data Analysis**:
   - Integrated *in silico* – *in vitro* data analysis
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**PSA SECRETION SCREENING TOOL: Vinclozolin and metabolites (1)***

Effects of Vinclozolin and M1 and M2 metabolites;

*Lorenzetti et al., unpublished data*
Effects of Vinclozolin and M1 and M2 metabolites upon DHT-cotreatment;

*Lorenzetti et al., unpublished data*
Assessment of the internal concentration of the chemicals in LNCaP cells and their intracellular distribution

*Lorenzetti et al.*, unpublished data
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questions are up to you!