

Symposia

(S 01 to S 23)

Early Diagnosis and Management of Prostate Cancer

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Prostate cancer is a significant cause of morbidity and mortality in the United States and Europe. It is the second most common cancer in men in the European Union, with 85,000 new cases and approximately 40,000 deaths each year. The natural ageing of the population, combined with the continued and widespread use of improved diagnostic tests such as serum prostate specific antigen (PSA), are resulting in an increase in the numbers of men diagnosed with localised prostate cancer. Screening to identify organ-confined disease has provoked much public and scientific attention and there is intense debate about its role in improving men's health. While there are strong advocates of screening, the findings from most reviews of the scientific evidence conclude that it is insufficient, at present to recommend routine population screening because of the lack of evidence that this would improve survival and the quality of men's lives. Particular concerns in these reviews relate to the lack of knowledge about the natural history of screen-detected disease, and the lack of evidence about the effectiveness of treatments.

In particular, to date, whilst the recent Scandinavian SPCG-IV randomized controlled trial of watchful waiting versus radical prostatectomy has demonstrated a survival benefit as well as reduced progression in men receiving surgery versus no treatment for clinically localized prostate cancer, no survival advantage has been shown to date in screen-detected cases for any of the major treatments (radical prostatectomy, radical radiotherapy including brachytherapy and "watchful waiting" otherwise known as "Active Monitoring" or "Active

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Surveillance with delayed intervention"). Each can result in damaging iatrogenic complications and outcomes, including various levels of incontinence and impotence for radical interventions and anxiety relating to the presence of cancer in untreated patients. Furthermore, recent observations from the Prostate Cancer Prevention Trial in the US have revealed that the incidence of the disease is considerably higher than expected at low PSA levels, with a significant risk of over-detection and over-treatment in the majority of screen-detected cases.

Despite these uncertainties, opportunistic screening for prostate cancer is widespread in many countries. Two randomised trials of screening are ongoing ; the Prostate Lung and Colorectal and Ovarian Trial (PLCO) in the USA and the European Randomised Screening for Prostate Cancer (ERSPC). There are two current trials of localised prostate cancer treatments: the Prostate Testing for Cancer and Treatment study (ProtecT) in the UK and the Prostate Cancer Intervention Versus Observation Trial (PIVOT) in the USA.

Whilst controversies persist in the management of localized disease, many advances have been made in the treatment of locally advanced, as well as metastatic prostate cancer. Improved technology and better understanding of the biology of prostate cancer have allowed new combination treatment strategies to be developed. Sophisticated radiotherapy combined with androgen suppression appear to improve survival in locally advanced disease, and fine tuning of interventions allow protection of the skeleton and improved palliation in metastatic and hormone refractory disease, using novel chemotherapeutic agents and bone resorption inhibitors such as bisphosphonates.

There is a considerable paradigm shift in the general management strategy for prostate cancer, from the development of active monitoring programmes for low-risk tumours, to the conversion of previously debilitating advanced disease into a chronic, manageable condition with improved outcomes and quality of life for our patients.

Microarray-based molecular profiling of advanced and hormone-refractory prostate cancer

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EU FP6 PRIMA (Prostate Cancer Integral Management Approach) VTT Technical Research Centre of Finland and University of Turku, Finland ; University of Cambridge, UK; Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France ; Tampere University of Technology, Tampere, Finland; Radboud University Nijmegen Medical Centre, The Netherlands

There are few treatment options available for hormone-refractory and metastatic prostate cancer, largely as a result of incomplete understanding of the molecular mechanisms underlying the disease progression. In order to characterize clonal selection mechanisms leading to the failure of androgen deprivation therapy, we have analyzed a unique sample set of 19 advanced prostate cancers, consisting of hormone-refractory prostate cancers (HR-PRCA) and untreated advanced tumors using oligo-CGH and gene expression microarrays. In addition, publicly available existing gene expression data from normal prostate tissues and untreated prostate cancers were used as a reference to validate the relevance of the gene targets identified here.

The genetic changes identified by oligo-CGH in the two groups were partly overlapping, but HR-PRCA also displayed many unique alterations (such as gains/amplifications at Xq12, 1q21-q32, 2q31-q33, 3q21-q28 and deletions at 15q14-q22 and 15q24-25). A novel data integration method, DNA/RNA Outlier Analysis, was used to identify the target genes for the genetic changes. Several outlier genes were identified as targets for genomic amplifications and deletions in both sample groups, such as AR as a target gene for Xq12 amplification in HR-PRCA, and PTEN as a target for 10q23 loss, as well as several other novel genes at these and other loci. Based on all the identified outlier hits, Wnt signaling pathway showed the most significant enrichment in both sample groups indicating that deregulation of this critical pathway in prostate cancer occurs through genome-level alterations.

In summary, we have applied here an integrative microarray based analysis on gene copy number and expression of advanced prostate cancers, which has led to the identification of several novel targets with putative relevance in hormone-refractory prostate cancers. Ongoing functional studies will further clarify their therapeutic potential.

Role of estrogen receptor β in the pathogenesis and progression of prostate cancer

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In earlier stages, prostate cancer (PC) depends on androgens and its growth can be usually controlled by androgen blockade. However, androgen-ablation therapy often induces androgen-independent PC, which is generally characterized by an increased invasiveness. Recently, we found that the testosterone derivative, dihydrotestosterone (DHT) inhibits PC cell migration through an androgen receptor (AR)-independent mechanism. This effect has been linked to the DHT metabolite 5 α -androstane-3 β ,17 β -diol (3 β -Adiol), which is unable to bind AR, but interacts with the estrogen receptor beta (ER β). 3 β -Adiol inhibits cell migration of PC cells through the activation of the ER β signaling, while, surprisingly, estradiol is not active, suggesting the existence of different pathways for ER β activation in PC cells. The inhibitory effects of 3 β -Adiol on PC cell migration appear to be mediated by an overexpression of E-cadherin, a protein known to be capable to reduce metastasis formation of breast cancer and PC cells. In fact, 3 β -Adiol is not active on PC cells in which E-cadherin expression has been silenced using a selective siRNA.

To further investigate the growth properties of PC cells in vivo, we implanted DU145 cells stably transfected with AR (DU145-AR) and DU145-mock s.c. into nude male mice to characterize their receptor state. Tumor growth was followed up to 25 days. The DU145-mock xenografts kept an exponential growth in such time frame ; castration only marginally influenced tumor growth. Interestingly, testosterone seemed to be able to reduce the growth of the tumors generated by DU145-mock suggesting that an androgenic metabolite (3 β -Adiol ?) may be responsible for this effect. On the other hand, tumor growth of DU145-AR xenografts was significantly lower and further inhibited by androgen deprivation. After testosterone replacement, the growth rate was restored to that of DU145-AR xenografts in intact animals, suggesting that the molecular changes present in the stably transfected DU145-AR cells influence their growth properties in in vivo systems.

The present data demonstrated that 1) testosterone may exert an estrogenic effects downstream in the catabolic process present in the prostate; 2) the estrogenic effect of

testosterone derivatives (ERbeta-dependent) inhibits cell migration, although it is apparently different from that linked to estradiol on the same receptor and may be protective against PC invasion and metastasis. These results correlate with the clinical data reporting that alterations in gene coding for 3beta-HSDs (the enzymes responsible for 3beta-Adiol formation) are strongly correlated with hereditary PC.

S 04

Benign prostatic hyperplasia – current management options and future prospects

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No abstract provided.

S 05

Germ cell cancer : clinical applications of gene expression profiles

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Recent years have seen an explosive increase in gene expression studies because of the availability of high-throughput methods, in particular microarrays (cDNA- or oligonucleotide- arrays). DNA chips - as the microarrays are popularly called – enable the investigator to evaluate relative expression of thousands of genes simultaneously in one experiment. This ingenious technology has provided researchers with an ocean of new information concerning global gene expression in many types of normal cells or tissues, and changes of their gene expression profiles in diseases, including cancer. The germ cell cancer research field has also benefited from these studies. Among the most important findings, to name just a few, are :

- high expression of pluripotency genes and stem-cell related factors in the pre-invasive carcinoma in situ (CIS) and embryonal carcinoma (EC) and EC-derived cell lines ;
- construction of new expression-based classifiers allowing to predict the predominant histological component of nonseminomatous tumours (nonseminomas) ;

- detection of first genes that are allow to distinguish very early seminomas from nonseminomas ;
- discovery of new gene expression markers of rare germ cell tumours that do not originate from CIS (infantile tumours or spermatocytic seminomas of elderly men) ;
- discovery of new markers applicable for clinical diagnosis of CIS, including CIS cells found in semen samples, e.g. TFAP2C (AP2 gamma).

Some of these findings are currently tested in clinical studies and begin to benefit patients.

S 06

Fetal androgen disruption and chronic adult germ cell apoptosis - a model for the testicular dysgenesis syndrome

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No abstract provided.

S 07

Fertility after testis cancer

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Testicular cancer (TC) is the most common cancer in men 20 to 35 years of age. Its prognosis is excellent and TC represents a model of curable cancer (cure rate exceeding 95%). For long-term survivors, often in the reproductive age, fertility preservation is a major preoccupation.

Epidemiological, histological and clinical data point out a common aetiology between testicular germ cell cancer, male genital abnormalities (such as cryptorchidism) and conditions

related to male reproductive health (such as infertility). More than half of the patients with TC already have testicular impairment before orchiectomy. The degree of spermatogenic dysfunction is higher than what can be explained by local tumour effect and by a general cancer effect. Histological abnormalities are frequently observed in the contralateral testis.

Treatments such as cytotoxic chemotherapies and radiotherapy are associated with significant gonadal damage in men. Cisplatin-based chemotherapy for TC results in temporary azoospermia in most men, with a recovery of spermatogenesis in about 50% of the patients after 2 years and 80% after 5 years. A cumulative dose of cisplatin at 400 mg/m² is predictive of the occurrence of long-term effects on sperm production. Second line chemotherapies may contain alkylating agents that are damaging agents for spermatogenesis.

The germinal epithelium is very sensitive to radiation-induced damage: Testicular doses of less than 0.2 Gy have no significant effect on FSH levels and sperm counts. Doses above 0.2 lead to a dose-dependent increase in FSH and reduction in sperm concentration. The time to recovery, if it occurs, is likely to be dose dependent. It is considered that doses of 1.2 Gy and above are at high risk of definitive impairment of spermatogenesis. Limitation of the radiation field to the para-aortic field, total radiation dose less than 30 Gy and testicular shielding are ways to limit radiation-induced impairment of fertility.

Information and counselling about semen cryopreservation, chance of recovery of spermatogenesis and current available methods of ART are mandatory. Cryopreservation of spermatozoa is a simple and practical approach to preserve fertilizing potential, and its proposal is part of good clinical practice. Wait-and-see strategies and ways to decrease morbidity of treatments should be strongly promoted and prospective trials comparing patients with and without cytotoxic treatment should be developed to ascertain the extent to which treatment affects long-term fertility. Cooperation between oncologists, urologists and andrologists on this topic remains essential.

S 08

Cryptorchidism – relaxins and other factors

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Cryptorchidism is a one of the most frequent congenital

abnormalities with recorded frequency of 3-4% among the newborn boys. Before sex determination both female and male embryonic gonads are located in the same high abdominal position. The developing testes are migrating through a multiphase process of testicular descent (TD), first into low abdominal position and then into developing scrotum. Critical role in TD belongs to the proper differentiation and growth of gubernacular ligaments. Analysis of the mouse mutants revealed a number of genes involved in this process. INSL3 (insulin-like 3) is a peptide hormone of relaxin-insulin family, expressed in Leydig cells in testis. INSL3 signals through its cognate G protein-coupled receptor LGR8, which is expressed in gubernacular cells and controls the transabdominal phase of TD. The inguinoscrotal stage of TD is believed to be mediated mainly by androgens. Several transcription factors, such as Hoxa10, Hoxa11, and Desrt, caused cryptorchidism in mice, suggesting an involvement of additional signaling pathways in TD. Using different mouse transgenic lines we have analyzed the regulation and interactions between these genes. The mutation analysis of INSL3 and LGR8 genes in human patients with testicular maldescent revealed mutant alleles present exclusively in affected patients. A unique missense mutation (T222P) in the ectodomain of the LGR8 receptor has been identified exclusively in patient group in some populations. The expression analysis of the mutant protein revealed that the mutation severely compromised receptor cell membrane expression. Consecutively, we have also shown compromised physiological properties of the mutant INSL3 variants identified previously in cryptorchid patients. Thus, mutations in the INSL3 and LGR8 genes might be responsible for the etiology of some cases of cryptorchidism in humans.

S 09

The role of chromatin in the establishment of paternal epigenetic information during spermatogenesis

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It is now admitted that, in addition to genetic information, the spermatozoon conveys information, named "epigenetic", which is not encoded by the DNA sequence. Epigenetic information is known to be crucial for the development of the embryo.

In somatic cells, the epigenome conveys the epigenetic information, which is supported by the DNA methylation as well as by modifications of the genome packaging structures, or epigenome, which consists of the nucleosomal chromatin (DNA wrapped around somatic histones).

During post-meiotic maturation of the male germinal cells, or spermiogenesis, the epigenome is completely re-organised since most somatic histones are replaced by sperm-specific basic proteins, the protamines, which are responsible for the tight compaction of the haploid genome within the sperm nucleus.

Although this process has long been described, very little is known about the molecular events and factors involved. We have identified several chromatin modifiers, which could have a crucial role during post-meiotic chromatin re-organisation.

Moreover, it is now known that the sperm genome is not uniformly packed with protamines, and that some histones remain, as well as some other non-histones, non-protamines basic proteins. Our recent data suggest that specific regions of the genome could be associated with specific structures within the sperm nucleus.

The heterogeneity and specificity of this structure organising the sperm genome could convey specific paternal epigenetic information to the zygote, which could play a key role during development.

Finally, the implications of these data in the field of male infertility will be discussed.

spermatogonia. Germline inactivation of *Rara* results in a testicular degeneration comprising some, but not all, abnormal features observed upon dietary vitamin A deficiency, whereas germline inactivation of either *Rarb* or *Rarg* do not cause primary testicular defects.

A selective ablation of the $RAR\alpha$ gene in mouse Sertoli cells (*Rara^{Ser}-/-* mutation) yields testis defects identical to those generated upon inactivation of $RAR\alpha$ in the whole organism, indicating that all $RAR\alpha$ functions in male reproduction are Sertoli cell-autonomous. During postnatal testis development, the *Rara^{Ser}-/-* mutation abolishes cyclical gene expression in Sertoli cells. It also induces testicular degeneration features, as well as a delay in spermatogonial *Stra8* expression, two hallmarks of RA deficiency. Thus, our data demonstrate that $RAR\alpha$ is a master regulator of the Sertoli cell cycle and plays important paracrine functions in spermatogonia differentiation and survival of germ cells. However, ablation of all three RARs in Sertoli cells does not induce an arrest in spermatogonia differentiation and does not abolish *Stra8* expression, as does vitamin A deficiency. These phenotypic differences point to a cooperation between distinct RA signalling pathways mediated by $RAR\alpha$ in Sertoli cells and by $RAR\gamma$ in spermatogonia during spermatogenesis.

Additionally, ablation of all three RXR (α , β and γ isotypes) in Sertoli cells does not recapitulate the *Rara^{Ser}-/-* mutant phenotype, providing thereby the first evidence that $RAR\alpha$ exerts functions in vivo independently of its heterodimerization with a RXR.

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Retinoic acid signalling in the testis

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Development and functioning of the mammalian seminiferous epithelium, require a complex assortment of hormones and cytokines. Amongst these, retinoic acid (RA), the active metabolite of vitamin A (retinol) regulates spermatogonia differentiation and spermatid adhesion properties. RA acts through binding to nuclear receptors, the Retinoic Acid Receptors ($RAR\alpha$, β and γ isotypes), which are ligand-dependent transcriptional regulators. In general, RAR transduce the RA signal in the form of heterodimers with REXINOID RECEPTORS, $RXR\alpha$, β and γ . Each RAR is detected in a specific cell-type of the seminiferous epithelium: $RAR\alpha$ in Sertoli cells, $RAR\beta$ in round spermatids and $RAR\gamma$ in A

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Connexins – relevance to rodent and human spermatogenesis

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Gap junctions contain channels that connect neighbouring cells and constitute the basis for direct intercellular communication via exchange of ions, second messengers and metabolites. Each channel consists of two hemichannels, called connexon, which are formed by the hexameric assembly of protein subunits, connexins (Cx), a family of highly related proteins, that consist of at least 21 members in human and 19 in rodents. In the testis, gap junctional intercellular communication is well known between interstitial Leydig cells and endothelia of blood vessels, and within the seminiferous epithelium

between Sertoli cells, and between Sertoli cells and germ cells.

In rodent testis, six isoforms have been demonstrated so far. Cx37 was solely found in blood vessel endothelia, while Sertoli cells express Cx 26, Cx32, Cx33, and Cx43. Germ cells are positive for Cx31 and Cx 43. In the testis, Cx 43 seems to be the most important and predominant connexon. In the seminiferous epithelium, it is first expressed together with the pubertal terminal differentiation of Sertoli cells and its expression depends on the seminiferous epithelial cycle. The importance of Cx 43 to gametogenesis is indicated by severe depletion of germ cells in prenatal male and female mice lacking the Cx43 gene. Postnatal spermatogonial proliferation is impaired in Cx43-null mutants, and this defect can not be restored by insertion of Cx32 or Cx 40 coding regions into the respective region of Cx43^{-/-} mice. It is impossible to evaluate the physiological role of Cx 43 or Cx 26 by knockout mouse models, because Cx43 knockout leads to altered cardiac morphology and perinatal death, and Cx 26 deficient mice die on embryonic day 11 because of placental deficiency.

In order to circumvent perinatal lethality and pleiotropic effects, we generated a mouse line that carries the floxed connexin 43 coding region flanked by loxP recognition sites for the Cre recombinase. Sertoli cell specific deletion was achieved by crossing these floxed mice with mice harbouring the transgene under control of Sertoli cell specific AMH gene. First data show, that these Sertoli cell specific Cx 43 knockout mice show a prepubertal arrest of spermatogenesis at the level of spermatogonia indicating, that Cx43 expression plays the key role in the initiation of spermatogenesis, which is not replaced by the up-regulation of other Cx's. Cx26 seems to be restricted in the apical region of rodent seminiferous epithelium, i.e. in spermatids and/or Sertoli cells. Cx 33 is a member of the gap junction showing several specialities. In contrast to most other Cx's which are also expressed in other tissues, Cx 33 expression seems to be restricted only to the testis. It was shown to inhibit the expression of Cx33 and to reduce Cx43. Its functional role is believed to limit the capacity of cells to create functional channels by enabling formation of heterotypic channels with other cells. The Cx33 gene, which is mapped to the X chromosome in the rat and mouse genome has no orthologs in the genome of humans or any other mammals, such as dog, cattle, pig, horse, and marmoset monkey, which have been investigated so far.

In the human Cx43 and Cx26 expression was intensely investigated. As in rodents, Cx43 is firstly found during pubertal Sertoli cell terminal differentiation and shows stage dependent expression in adult normal seminiferous epithelium. Cx26 is weakly expressed and located at inter Sertoli cell junctional sites. Both Cx's have been found to be functional Sertoli cell markers, indicating the state of Sertoli cell differentiation in testes of infertile patients showing spermatogenic impairment. Sertoli cells in seminiferous tubules showing an arrest of the seminiferous epithelium reveal a persistent AMH expression together with an aberrant diffuse cytoplasmic Cx26 staining, and absence of Cx43 expression indicating a prepubertal state of Sertoli cell population without functional intercellular communication. This is also true for Sertoli cells associated

with preinvasive testicular intraepithelial neoplasia (TIN), syn. : Carcinoma in situ (CIS) which is known to be the precursor of germ cell tumours such as seminomas and non seminomas with the exception of spermatocytic seminoma. In TIN tubules Sertoli cells show a progressive down-regulation of Cx43 indicating the loss of intercellular communication between Sertoli cells and between Sertoli cells and preinvasive tumor cells. Aberrant gap junctional intercellular communication and/or loss of connexin expression has been demonstrated to correlate with neoplastic transformation in several human tissues such as prostate, breast, lung, and brain. Thus the loss of gap junctional intercellular communication might lead to uncontrolled mitotic division of CIS cells and finally to solid invasive neoplasia.

References

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Databases and data mining in studies on spermatogenesis

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No abstract provided.

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Human epididymal proteins: regulation and interactions with spermatozoa

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Vasectomy is the third most common contraceptive method

and the demand for surgical vasectomy reversal (vasovasostomy) is increasing. Some consequences of vasectomy on human epididymal physiology will be presented. Vasectomy disturbs gene expression in the human epididymis. Vasectomy modifies the pattern of expression of P34H, a protein expressed in the human corpus epididymidis, added to the sperm surface during maturation and involved in zona pellucida binding. While P34H is expressed in the corpus epididymidis in normal men, its expression is restricted to the proximal caput epididymidis. This may affect the fertilizing potential of ejaculated spermatozoa of some vasovasostomized men.

In fact, an important proportion of vasovasostomized men are characterized by spermatozoa lacking the P34H protein. Although the expression of P34H in the epididymis is relocated under vasectomy, HE1/NPC2, a gene expressed all along the human epididymis, is down-regulated under vasectomy. Western blot analyses show that many vasovasostomized men are characterized by high HE1/NPC2 levels on spermatozoa when compared to fertile donors. HE1/NPC2 association with sperm from vasovasostomized men is not correlated to low motility per se as spermatozoa from asthenospermic men have similar levels of HE1/NPC2 as in normal fertile semen samples. Spermatozoa from vasovasostomized men with high amount of HE1/NPC2 are characterized by higher concentration of cholesterol and more lipid raft domains. Knowing the effect of cholesterol on sperm physiology, this may affect the fertilizing ability of the male gametes. HE1/NPC2 is secreted in different glycoforms by different tissues of human male reproductive tract. These forms are due to variation in N-glycosylation and only the deglycosylated form is associated with spermatozoa from some vasovasostomized men.

Compared to normal men, seminal plasma of vasectomized men is characterized by a major decrease in immunodetectable HE1/NPC2 without change in the glycosylation pattern. Following surgical vasectomy reversal, seminal plasma HE1/NPC2 is found in similar amount to the ones characterizing normal men. Considering the potential role of HE1/NPC2 in cholesterol transport during sperm maturation, unusual high levels of this protein associated with spermatozoa of vasovasostomized men may reflect epididymal sequelae occurring when the vas deferens is obstructed. Taken together, our results show that vasectomy affects gene expression along the human epididymis. These changes have consequences on some biochemical characteristics of ejaculated spermatozoa of vasovasostomized men. Thus, sequelae to the human epididymis under vasectomy may not be reversible in all men following vasovasostomy. This may explain the discrepancy between surgical success of vas deferens reanastomosis and fertility recovery.

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Human epididymal proteins - secretion, absorption, and luminal fluid composition

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Sperm membrane dynamics : structure and function

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Sperm plasma membranes are unusual for their high content of unsaturated phospholipids and their compartmentalisation into at least 5 recognizable domains, each with specific and overlapping lipids and glycoproteins. Elucidating how these domains are created and maintained over relatively large distances against the randomising forces of diffusion is central to understanding membrane remodelling events during sperm maturation and capacitation. Foremost amongst remodelling processes is repositioning of specific components from areas of the spermatozoon where they are 'inactive' to regions where they become 'active', either as a result of processing (e.g. endoproteolysis) or because they form complexes (e.g. lipid rafts) with new membrane components.

To understand the supramolecular structure of the sperm's plasma membrane we have applied a range of quantitative, high resolution microscopy techniques to investigate the dynamics of lipid and protein diffusion in live spermatozoa (boar, ram, bull and mouse) at different stages of development. The methods include atomic force microscopy (AFM), scanning ion conductance microscopy (SICM), fluorescence recovery

after photobleaching (FRAP), fluorescence loss in photobleaching (FLIP), single particle fluorescence imaging (SPFI) and single molecule tracking (SMT). AFM revealed significant differences in membrane topography between the acrosome, postacrosome and identified a new region within the equatorial segment, designated the subsegment (EqSS). The EqSS is enriched in Hsp70 and constitutively phosphorylated proteins and is assembled during epididymal maturation.

FRAP analysis using lipid reporter probes demonstrated significant differences between the sperm head and tail domains that, surprisingly, were largely unaffected by lipid peroxidation from oxygen free radicals. Combined FLIP and SPFI analysis suggested the presence of a molecular 'filter' rather than an absolute diffusion barrier between the acrosomal and postacrosomal domains. This has been substantiated by SMT although the nature of the probe and target molecule has to be taken into account. Thus, sperm membranes can respond rapidly to agonists in external fluids, be they in the epididymis or female reproductive tract or artificial media, by remodelling the composition of specific membrane domains and by assembly/disassembly of multimolecular complexes that transduce signals across the membrane. This hierarchy of responses at each stage of maturation and capacitation ensures that only fully competent spermatozoa reach the site of fertilization in vivo.

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Sperm calcium signalling - nature and function

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Prior to fertilisation a human sperm must complete a series of tasks in order both to reach the egg and to be competent to fertilise when it arrives. These functions must be activated discretely and must be timed appropriately. They are dependent entirely upon 2nd messenger signalling, particularly $[Ca^{2+}]_i$. Compared to somatic cells, spermatozoa appear extremely simple, reduced to only those components necessary to achieve their single, specific purpose.

However, recent studies have transformed our understanding, revealing the spermatozoon to be a complex, highly-organised and tightly-regulated cell. Sperm express their own, apparently unique, 'toolkit' of Ca^{2+} homeostatic mechanisms. Regulation

of Ca^{2+} -fluxes at the plasma membrane is essential for activation of acrosome reaction upon binding of the zona pellucida and for chemotactic responses mediated through the olfactory receptors that have recently been found in human sperm.

In contrast, progesterone apparently regulates motility by controlling mobilisation of Ca^{2+} stored inside the cell. It has become clear that sperm show surprising sophistication in their ability to generate complex spatio-temporal $[Ca^{2+}]_i$ signals and use them discretely to regulate the different activities of the cell, control their 'behaviour' and maximise their chance of success.

Significant progress in understanding the functioning of sperm and the way in which their activities are regulated opens up new possibilities for studying fertilisation, for treating male sub-fertility and for development of new contraceptive strategies.

S 17

Definition and epidemiology of premature ejaculation

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No abstract provided.

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Pathogenesis and diagnosis of premature ejaculation

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Premature ejaculation (PE) is a symptom rather than a disease. In fact, it can be caused by short frenulum of the prepuce, penile hypersensitivity and reflex hyperexcitability, prostatitis

and thyroid hyperfunction. Some researchers believe that PE is not a psychological disorder but a neurobiological phenomenon. This is a very interesting pathophysiological explanation, based on the role of serotonergic system in the control of ejaculation. As a matter of fact, the effectiveness of serotonergic antidepressants demonstrates that central neurotransmission is involved in ejaculatory control, but not that serotonin hypoactivity is the cause of PE, as suggested. In fact, many psychological disturbances provoke a neuroendocrine imbalance.

On the other hand, the classic psycho-sexological approach affirms apodictically that PE is a psychosexual disorder, which is 'all in the mind' with a psychogenic aetiology and pathogenesis that must be treated with psychotherapy.

The possibility that PE may involve marital problems should always be considered, even though it may be difficult to discern whether the couple's troubles are the cause or the effect of PE. Whenever possible, sexual symptoms should be assessed in the context of the couple. For this reason, it is essential to compare the male's description of the symptom with that of his partner. Perception of penetration time is in fact very subjective.

The physical examination of the patient with PE is usually normal: pathological findings are unlikely to be associated with this condition. However, penile biothesiometric measurement of the penile shaft, the glans penis and the mid scrotum has been proposed as a useful method to evaluate and qualify penis sensitivity, but denied by others. The biothesiometer is a vibrating device with a fixed frequency of 50 Hz and variable amplitude which is placed on these genital areas. The patient is asked to inform the examiner of the first sensation of vibration as the amplitude is slowly increased.

A possible flow chart of PE diagnosis is proposed. It should include prostate evaluation by transrectal ultrasonography and standardized Meares & Stamey protocol (1968). Urethral and midstream bladder urine, expressed prostate secretions by prostate massage, and post-massage urine samples are collected, examined microscopically, and cultured bacteriologically. Prostate inflammation is diagnosed if 10 or more white blood cells per high power field are present in the expressed prostate secretions. Non-bacterial prostatitis is defined by evidence of prostate inflammation together with negative urine and prostate fluid cultures. Prostate infection is defined by a colony count 10 times greater in the expressed prostate secretion or post-massage urine sample than in the urethral urine sample. The presence of Chlamydia Trachomatis, Trichomonas vaginalis, Mycoplasma hominis, Candida species, and Ureaplasma urealyticum should be carefully checked.

Negative subjects should be further studied for the possible presence of hyperthyroidism and, in selected cases, neurological diseases.

Management of premature ejaculation

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Antiviral defences of the testis

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Viral infection of the testis can have damaging consequences for the fertility and general health of the individual : it may disrupt the reproductive and endocrine functions, as illustrated by mumps virus and HIV infection in human, and potentially lead to testicular cancer (although the latter remains to be formally proven). It may also participate to the spreading of viruses from host to host through contaminated semen. Moreover, viral infection of the germ cells may result in the transmission of virus-induced mutations to subsequent generations.

Acquired immune reactions are normally suppressed in the testis in order to prevent germ cells destruction. We seek whether the testis possesses innate defense mechanisms to counteract viral attack and limit harmful infiltrations of specialized immune cells. Our research was initiated in the rat since there are no documented viral infections of the testis in this animal. Interferons (IFNs), discovered through their ability to "interfere" with viral replication, represent the main component of the cell antiviral response. These cytokines act in an autocrine/paracrine manner by inducing the expression of various proteins (the so-called IFN-induced proteins), the three best characterized in term of antiviral activity being the 2'5' oligoadenylate synthetase (25OAS),

the double strand RNA activated protein kinase PKR and the Mx proteins. IFNs and IFN-induced proteins expression was investigated in different rat testicular cell types in both basal conditions and following in vitro viral stimulation (Sendai virus, an RNA virus of the paramyxovirus family). The protection of the germ cells in the seminiferous tubules is of prime importance because their infection may result in the total destruction of the germ line or the dissemination of the virus or viral DNA to all germ cell classes, including the spermatozoa themselves. Spermatogonia are one of the most important potential targets because unlike the meiotic and postmeiotic germ cells, they are not isolated from the molecules and agents produced by the interstitial compartment.

The basal tubular position of these germ line precursors, a priori, exposes them to the risk of contamination in cases of viral infection. Despite the high potential risk of infection of spermatogonia, these cells do not constitutively express biologically active IFNs and they respond only weakly to stimulation by the Sendai virus in vitro. Moreover, they only weakly express the antiviral protein Mx and do not express 2'5'AS or PKR. Similarly, early spermatids and pachytene spermatocytes, although expressing low levels of IFNs in basal conditions, respond very weakly if at all to exposure to the Sendai virus and do not produce any of the 3 IFN-induced antiviral proteins tested (2'5'AS, PKR and Mx).

It was therefore suggested that the germ cell antiviral protection system may involve mainly the somatic peritubular and Sertoli cells within the seminiferous tubules and the somatic Leydig cells and macrophages within the interstitium, all of which have subsequently been shown to express both high levels of IFNs and IFN-induced proteins in response to viral infection. Of all the testicular cell types tested, Leydig cells and Sertoli cells have by far the strongest antiviral potential. Thus, in the rat testis, a first line of defense against viruses arriving in the bloodstream is represented by Leydig cells and testicular macrophages, whilst a second line of defense involves the myoid cells lining the seminiferous tubules and Sertoli cells.

However, the situation may vary in between species as the first results obtained in human Leydig cells show lack of IFN production when exposed to a range of viral stimuli, including mumps virus and the RNA virus-like, synthetic double strand RNA poly IC. Despite this, virally stimulated Leydig cells over-express two antiviral proteins, the 25OAS and MxA protein. To fully apprehend the innate immune status of the human testis, the antiviral capabilities of all testicular cell types will need to be tested.

Viruses and semen

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Most sexually transmitted viruses can also be found in male or female genital secretions and detected by cell culture or genomics. HBV, HSV, CMV, HHV-8, HPV, HTLV and HIV have been studied in human semen. Some other viruses can be detected in semen using new improved molecular biology techniques, but they are poorly transmitted during sexual intercourse. This is particularly true for HCV. Many other viruses that are probably non-pathogenic may be detected in semen. All viruses involved in viremia or associated with white blood cells can be, at least in theory, present in semen.

The viruses in semen, can be present as free virus particles in the semen plasma, as infected cells (with productive or latent infections), and in the spermatozoa themselves. The spermatozoa are unlikely to harbour productive infections, but they may have latent infections and virus reactivation cannot be excluded. This has been shown for human endogenous retroviruses (HERV) and remains a discussed possibility for HBV. The infected cells are usually lymphocytes, monocytes or polymorphonuclear cells. An increase risk of virus shedding is associated with the presence of these cells in semen and when there is genital inflammation or infection.

Free virus particles in the semen can come from the blood by passive diffusion during the viremia phase of infection or from local virus replication. The characteristics (genome sequence) of virus produced by local replication in semen are often different from those of viruses in the blood, reflecting compartmentation.

Semen is screened to detect viruses with pathogenic potential so as to limit sexual transmission and the risk of transmission during medically assisted procreation (MAP). Screening is based on virus acquisition risk factors, clinical signs, systematic or oriented biological screening. Vaccines, when available, are then used to avoid transmission to the partner. Antiviral treatments are becoming more and more efficient and can cure a patient by eradicating virus from his/her body in some cases (HCV for example). But they usually only limit virus replication or keep it below the level needed to control clinical evolution. These antiviral treatments can reduce the virus concentration in the semen and thus the risk of transmission, depending on their ability to diffuse into the male genital tract. The assays used to detect virus in semen have improved greatly in recent years and are now very sensitive and less influenced by

semen PCR inhibitors. However, a negative result does not mean that virus is absent, since there is always a detection threshold in all assays of virus genomes.

Sperm processing techniques can further decrease the risk of transmission from patients that may have residual virus in their semen (eg. HIV) by eliminating semen plasma and non-spermatozoa cells and by keeping only spermatozoa for MAP. These methods have proved to be effective for treating semen containing HIV-1 and HCV and should be just as effective for removing other viruses.

Nevertheless, the way many viruses are shed into the semen has not yet been characterized, especially during incubation phase or when the infection is asymptomatic or acute. This should be taken into account in the future management of the risk of transmission of viral diseases.

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Assisted reproductive technology in couples with HIV-infected male partners

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The development of highly active antiretroviral therapy (HAART) over the last 10 years has transformed the prognosis of patients infected with human immunodeficiency virus type-1 (HIV) living in the developed world. HIV has been reclassified as a chronic disease with good life expectancy and quality and many heterosexual couples, where one or both partners are infected, are now considering parenting as a realistic option

In a heterosexual couple where the male partner is infected, unprotected intercourse carries a risk of transmitting HIV to the uninfected female partner and potential child of 0.1% per act of intercourse. Unfortunately viral load in semen correlates poorly with that in serum and men on antiretroviral medication with undetectable viral loads can still have detectable virus present in semen.

Sperm washing is a specific risk-reduction option available to HIV serodiscordant couples where the male is infected trying to conceive. Semen is centrifuged in a density gradient to separate live sperm, which does not carry HIV, from seminal plasma and non-germinal cells which may carry virus and then inseminated into the female partner at the time of ovulation. If a couple have additional fertility issues, sperm washing can be combined with ovulation induction, in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI). In order to ensure the final preparation used in clinical practice is free of HIV and safe to use, an aliquot of washed sperm is tested for detectable HIV RNA prior to the sample being used for treatment. A nucleic acid-based sequence amplification (NASBA) or similar commercial PCR assay can be used.

The clinical program for sperm washing at The Chelsea and Westminster Hospital was established in 1999 and has performed the largest series on treatments in the UK. 106 couples have undergone 293 cycles of IUI with a live birth/ongoing pregnancy rate per cycle of 10.2% and clinical pregnancy rate of 14.3%. These figures are similar to national statistics for IUI using donor sperm and compare favourably to results reported from other centres in Europe with similar programs for serodiscordant couples. In terms of safety, there have been no seroconversions in either female partner or child on rigorous follow-up (HIV testing of female partner for up to 6 months after the last treatment with washed sperm) and 27 healthy children have been born to date through the IUI program alone (45 through the sperm washing program including IVF and ICSI cases). In our series, the cancellation rate due to a positive NASBA post wash was 3.3%.

Semen samples from 106 HIV-positive men undergoing sperm washing IUI were compared with those from a control group of 234 HIV-negative men undergoing IUI (Nicopoullos et al., 2004). Markers of HIV were assessed and although the majority of HIV-positive men had sperm parameters within the defined WHO 'normal' range, ejaculate volume, sperm concentration, total count, progressive motility and normal morphology were all significantly lower in the HIV-positive group compared to the HIV-negative controls ($p < 0.05$). There was a significant positive correlation between CD4 count and sperm concentration, total count and total and progressive motility and a significant negative correlation with normal sperm morphology of both raw and post preparation samples. There was no correlation between viral load, years since diagnosis, use of or duration of use of HAART with any semen parameter. This is the largest analysis of semen parameters in HIV-positive men to date.

We also analysed the factors which had an impact on IUI outcome in our first 140 cycles of IUI with sperm washing. Clinical pregnancy rate was significantly higher in cycles where the man had a low viral load (<1000 copies/ml; 29% versus 11%, $p=0.05$) and in cycles where the man was on HAART (27% versus 9%, $p=0.02$). CD4 count had no impact on IUI outcome. Although these data clarify the factors that influence IUI outcome, they do not shed light on the precise mechanism by which HIV and markers of HIV infection alter semen parameters and IUI outcome which should be the subject of further investigation. Our present advice is that the decision to start or stop medication should be not be based on concern for fertility but rest primarily on viral load and CD4 count parameters and health of the individual.

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Bacterial male genital tract infection and sperm quality

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The field of bacterial infection and sperm quality, stagnant for at least three decades, has changed from the nineties years. Two different panel of scientists evaluated this research area ending up with two independent consensus conferences. The first, of mainly urological background (NIH Chronic Prostatitis Collaborative Research Network), focused on prostatitis classification and only two categories (II and IV) were associated with male infertility (Krieger et al., 1999; Nickel et al., 2001). Sperm analysis was included among the optional tests and therefore it does not play a pivotal role. This justifies the contradictory data available on the influence of urogenital tract infection on sperm quality with a full array of effects reported which range from a negative impact on all main sperm parameters (density, motility and morphology) or on sperm motility only, to no sperm parameter alteration at all (see Weidner et al., 1999, for review).

The second task force, of uro-andrologic background (WHO Task Force on the Diagnosis and Treatment of Infertility (WHO, 1993), identified a diagnostic category affecting male reproductive function and fertility which was named male accessory gland infection (MAGI). In this classification, sperm analysis is viewed as a fundamental step of the diagnostic workup. In fact, MAGI is diagnosed in presence of one or more sperm parameter abnormalities (oligo- or astheno or teratozoospermia) associated with a combination of two or more of the following factors :

- Factor A : history positive for urinary tract infections and/or sexually-transmitted diseases and/or physical urogenital examination ;
- Factor B : expressed prostate signs of infection and/or inflammation ;
- Factor C : ejaculate signs of infection.

According to the NIH classification, seminal bacterial infection, corresponding to chronic bacterial prostatitis (category II, NIH classification), has a marginal epidemiologic role since bacterial prostatitis (acute or chronic) accounts for a low number of patients with prostate symptoms (5-10%) (see Weidner et al., 1999, for review). Recently, Li and colleagues reported a higher rate of bacterial infection (24.2%) in patients with prostatitis type IV (Li et al., 2004). On the contrary, MAGI has been reported to account for a wide range of frequency (1.6-

39.1%) of infertile patients according to the various infertility clinic settings (Comhaire et al., 1986; Andreeßen et al., 1993; Vicari, 2000; Diemer et al., 2003; Li et al., 2004).

In the course of urogenital bacterial infection, sperm quality is the final product of germ intrinsic properties (degree of virulence, bacterial load), time of interaction the microorganism and the involvement of one or more sex accessory glands. Some Gram Enterobacteriaceae, such as *Escherichia coli*, *Klebsiella* sp., *Proteus*, *Serratia*, *Pseudomonas* sp, etc., have been recognized as known prostate pathogens (category II, NIH classification) having a strong association with a clear positive clinical history (prior and/or recurrent urinary tract infection, sexually transmitted disease, congenital uro-genital abnormalities) and some uro-genital abnormalities at the physical examination. On the other hand, the only presence of some microorganisms is interpreted by some investigators as "probable" (when Gram-positive pathogens, such as *Enterococcus* sp, and *Staphylococcus aureus*, are present) or "possible" (when coagulase negative pathogens, such as *Staphylococcus*, *Chlamydia*, *Ureaplasma*, anaerobes are present) prostate infection. The major difficulty in interpreting microbiological findings is the presence of contaminating, indigenous microbiota, or of inhibitory substances known to be present in the prostatic secretions, as well as previous courses of antibiotics. Thus, the diagnosis of bacterial prostatitis may be confirmed by quantitative bacteriological cultures in the semen (growth of $>10^3$ pathogenic bacteria or $>10^4$ non-pathogenic bacteria in seminal plasma diluted 1:2 with saline solution) (Comhaire et al., 1980) or segmented cultures, i.e. four (Meares and Stamey, 1968) and/or two (Nickel, 1997) glass test.

In addition, underlying prostate and systemic chronic comorbidities have to be taken into account. Thus a negative impact on sperm quality may arise from one (or more) of the following mechanisms: 1) secretory dysfunction of one or more male accessory glands; 2) deterioration of spermatogenesis; and 3) (unilateral or bilateral) organic or functional sub-obstruction of the seminal tract. Gland secretory dysfunction represents the most relevant cause of negative impact of sperm quality. It induces an inflammatory response (leukocytospermia, reactive oxygen species and/or cytokines release, such as IL-1 β , IL-6, IL-8, TNF α ; and autoimmune processes) (Ochsendorf, 1999; Vicari, 2000; Vicari & Calogero, 2001; Weidner et al., 2002; Vicari et al., 2002; Diemer et al., 2003). These bioactive substances may persist even following successful treatment with antimicrobials, since the initial antioxidant capacity (mainly based on epididymal biological micronutrients of the seminal plasma) is progressively exhausted, thereby impairing sperm function by inducing DNA damage and/or apoptosis (Agarwal et al., 2003; Sanocka et al., 2003). Gland structural abnormalities may, in addition, play a negative role. In fact, infertile patients with MAGI and elevated bacteriospermia ($\geq 10^5$ CFU/ml) or with *Chlamydia* or *Ureaplasma urealyticum* infection (at urethral swabs after prostate massage) have a higher number of ultrasonographic abnormalities involving more glands (prostatovesiculopididymitis, epididymo-orchitis) (Vicari, 1999; Vicari et al., 2006). These patients show also to have an increased

inflammatory response and an impaired semen quality directly related to MAGI extension (prostatitis < prostato-vesiculitis < prostato-vesiculo-epididymitis) (Vicari, 1999) or a strong association with sperm abnormality (Bayasgalan et al., 2004).

In conclusion, sperm parameters represent the "end-target" of many possible pathophysiological mechanisms which may contribute to the onset of infertility related to urogenital infections. Therefore, despite an open debate with pros and cons on the role of MAGI in male infertility is going on, the andrologist should at least consider MAGI as a risk factor of male infertility (Bayasgalan et al., 2004), different from one country to another. Thus, MAGI may be or may become an effective cause of male infertility through the above-mentioned multiple pathophysiological mechanisms which in turn impair sperm function.

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