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PROJECT FINAL REPORT

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Name of the scientific representative of the project's co-ordinator[1],

Prof. Anders Arner

Department of Physiology and Pharmacology

Karolinska Institutet

SE 171 77 Stockholm

Sweden

Tel: +46 8 524 87221

E-mail: -Anders.Arner@ki.se

Project website address: www.INComb.eu

[1] Usually the contact person of the coordinator as specified in Art. 8.1. of the Grant Agreement.

4.1 Final publishable summary report

4.1.1 Executive summary

Urinary incontinence and the problems of urgency and frequency of micturition are conditions affecting a large number of European citizens. An “over active bladder” (OAB) due to involuntary bladder muscle contractions is the major cause of urgency incontinence. Although this and other lower urinary tract symptoms do not constitute life threatening disorders, they do have serious negative impact on the quality of life and major effects on health economy. Several therapies are used to treat urgency incontinence. However, currently, these treatments are imperfect or are associated with adverse side effects which reduce patient compliance. The INComb project, “Combating incontinence” (www.INComb.eu), was initiated, with the aims of establishing a better understanding of the pathogenesis of bladder overactivity, improving diagnostic tests and developing novel intervention strategies. The urinary bladder is a complex organ where different cell types and tissues influence the filling and emptying of urine. The project has addressed objectives related to the communication between the different cell types and has developed novel techniques to measure signalling components in urinary bladder tissue. Factors involved in OAB, mainly the communication between the inner lining of the urinary bladder (urothelium), the sensory nerves, the smooth muscle and other cell types were examined. “Interstitial cells” have been identified as modulating the contractile activity of the bladder wall and the project has analyzed their structure and localization in animal urinary bladders and in normal and diseased human bladders. The results describe their changes in different pathological conditions and have also revealed novel receptors on the interstitial cells that potentially can be used in diagnostics or for targeted therapy. The groups have examined the mechanisms and structural effects of botulinum toxin in the human bladder wall and provide new information regarding the mechanisms involved in the beneficial effects of botulinum toxin treatments in OAB. We have shown that TRPV1 and related receptors can be influenced by specific chemical compounds, suggesting a possible new therapeutic approach. New biomarkers associated with growth factors and novel links between bladder activity and pain signalling have been identified. INComb partners have also applied biobanking studies and analyzed protein expression in the bladder wall and genetic information directly linked to human bladder function. INComb participants have published 60 full scientific papers, 39 reviews, several theses and reports and 81 conference abstracts as the result of this project. Several further studies will be reported in the near future. Internal scientific meetings and an international conference, (“Urinary incontinence – from basic science to clinical practice”, Stockholm June 2011: a joint symposium between INComb, the EU project TRUST, the Swedish Enuresis Academy SEA and with support from the European Association for Urology) have been organized.

INComb scientists have performed extensive dissemination activities including interactions with patient organisations, television and news paper interviews. Several new mechanisms of potential therapeutic and diagnostic value are identified. INComb has in addition identified new and very interesting areas for future research. INComb is a group of research workers from several established and successful European clinical and basic research laboratories, all active in the field of lower urinary tract function but with different specialised skills. The centres in the consortium include Queen’s University Belfast; University of Surrey; University College London; Pfizer Limited; University of Amsterdam; Porto University; University of Zürich; Dr H. John at Winterthur Hospital, Switzerland; Lund University and Karolinska Institutet. The project is coordinated from Karolinska Institutet.

4.1.2 Project context and objectives

The INComb project has been studying the mechanisms involved in **urinary incontinence, urgency and frequency of micturition**. It has been estimated that more than 60 million people in the European union are affected, and although these conditions are not life threatening, they seriously affect quality of life of the individual. As a consequence, they produce major problems for individuals, health professions and society. These lower urinary tract symptoms are more prevalent in the older population, where 40% of individuals over the age of 70 are affected. Importantly, as people are generally living longer, the numbers of affected people are increasing. Only one in four of those who suffer from the condition have sought help and from those, only half currently receive treatment. Sadly, less than 3 in 100 persons treated regain long lasting normal bladder control. The mainly non-pharmacotherapeutic costs are thus an under-estimation of the financial magnitude of the problem. It has been reported that the annual financial implications of incontinence are very high and comparable to those of other chronic diseases, such as dementia or diabetes mellitus.

The primary clinical problem and major impact to the patient is urgency to void. Remarkably, despite the prevalence and costs involved, the mechanisms underlying urgency are currently unknown. In order to simply describe the condition, the term **"Overactive Bladder (OAB) Symptom Complex"** has been introduced. In many patients, urgency can be correlated with rises in the pressure, "detrusor overactivity", in the urinary bladder that can be detected by clinical urodynamical investigations. In other patients the urgency is not accompanied by detrusor overactivity, a condition that has been denoted "sensory urgency". The clinical condition can thus reflect alterations in both the sensory mechanisms and in the contractile function or activation of the urinary bladder muscle. Importantly, the complex and broadly defined clinical descriptions reflect that we still do not understand the nature of this important clinical condition.

Since the detrusor overactivity component of OAB is associated with bladder contractile activity, drugs affecting contractility could alleviate symptoms. Currently available pharmacological incontinence therapies have ranged from e.g. receptor antagonists to neuromuscular blocking agents and sensory blocking agents. As each of these therapies has varying degrees of side-effects, benefits are often outweighed and there are resulting issues with patient compliance. The INComb project was therefore aimed to drive the much-needed increase in our understanding of bladder function and dysfunction, with a clear focus on establishing mechanisms to alleviate bladder overactivity.

The general objectives of INComb were: (1) **obtain a better understanding of the mechanisms of bladder overactivity,** and **(2) improve diagnostics and develop intervention strategies,** specifically addressing the call HEALTH-2007-2.4.5-11

To address these objectives a combined approach including basic and clinical science is required and the INComb constellation was initiated with the specific goal to create a strong **platform for high quality translational research** between basic science and clinical practice. The beneficiaries included internationally established scientists from both clinical urology and experimental science. The project has also included cooperation pharmaceutical industry (Pfizer, Global Research and Development, UK).

The **INComb participants** were: Beneficiary #1- Karolinska Institutet, Stockholm (Prof. Anders Arner, Physiology; Prof. Peter Wiklund, Urology); Beneficiary #2-Queen's

University, Belfast (Dr. Karen McCloskey, Physiology); Beneficiary #3-University of Porto (Prof Francisco Cruz, Urology/Histology); Beneficiary #4-University of Amsterdam (Prof. Martin Michel, Pharmacology and Pharmacotherapy); Beneficiary #5 and #6 University of Zürich and Dr. H. John at Kantonsspital Winterthur , Zürich (Prof Caroline Maake, Anatomy and Prof H. John, Urology); Beneficiary #7-University College London (Prof. Clare Fowler, Neuro-Urology); Beneficiary #8-University of Lund (Prof. Bengt Uvelius, Urology) ; Beneficiary #9-Pfizer Ltd. (Drs Rachel McCoy and Gordon McMurray) and Beneficiary #10-University of Surrey (Prof Christopher Fry, Cell Physiology).

The project was coordinated by Karolinska Institutet (Prof. Anders Arner) with the senior administrative assistance of Dr. Cecilia Lövdahl. The objectives for the **management workpackage (WP1)** included the establishment of a set of management functions, routines for ethical issues and several dissemination activities including interactions with patient organisations and an international workshop "Combating incontinence: - from basic science to clinical practice"

The urinary bladder contains an inner lining (the urothelium), a muscle component (the detrusor smooth muscle), sensory and motor nerves as well as several other cell types, including a recently discovered interstitial cell localized in different regions of the wall. The process of filling the urinary bladder, storing the urine and emptying (micturition) are regulated by several regulatory systems - including nerve activity and local factors acting in the bladder wall. To understand the function of the normal urinary bladder and the changes in bladder overactivity, the S&T activities of INComb therefore focussed on key components in the bladder wall as depicted in the Figure below.

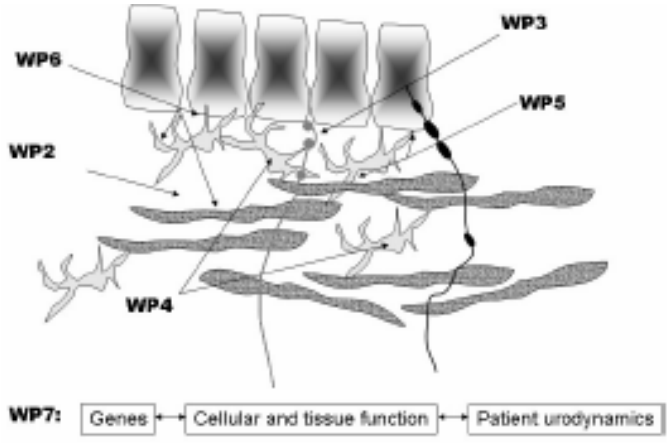

Figure: *The main cellular components of the bladder wall (urothelium, nerves, smooth muscle and interstitial cells) and the target areas for the different work packages.* ***WP2****: Modulator release;* ***WP3****: Sensory innervation and TRPV-signalling;* ***WP4****: Interstitial cell function in the bladder;* ***WP5****: Myogenic mechanisms and inter-cellular communication;* ***WP6****: Receptor signalling in the different components of the bladder;* ***WP7****: Correlation between urodynamics and genes.*

Workpackage 2 (WP2) aimed at examining the local signalling between the urinary bladder wall components with the objective to "**develop novel micromethods to determine intercellular signalling through various modulators originating from the bladder wall."**

Workpackage 3 (WP3) examined the components of sensory signalling with a focus on a group of cellular receptors (TRPV) with the objective to "**explore the therapeutic potential of bladder sensory innervation."**

Workpackage 4 (WP4) studied the properties of a recently discovered cell type in the bladder wall, the Interstitial Cells (IC). The objective was to "**describe the characteristics and function of a recently discovered novel cellular constituent of the bladder wall, i.e. interstitial cells, themselves a possible innovative therapeutic target."**

Workpackage 5 (WP5) focussed on the mechanisms by which the smooth muscle cells communicate in the bladder via direct electrical communication and explored local heat treatment as a potential therapeutic method. The objective was to **assess whether spontaneous activity generated in the bladder wall is amenable to therapeutic intervention by pharmaceutical or novel physical means."**

Workpackage 6 (WP6) examined the receptor signalling in different cell types in the bladder wall with the objective to: **"elucidate if cross-talk between different cellular signalling pathways is involved in OAB and can be manipulated therapeutically."**

Workpackage 7 (WP7) coordinated the experiments on human tissue and genetic samples. All work packages included combined studies of experimental animals and on human tissue sample and the WP7 specifically developed routines for sample handling and ethical aspects and coordinated biobanking approaches. The objective was to "**provide a suitable robust platform for experiments on human tissue and link clinical data to genetic information."**

4.1.3 Main S&T results/foregrounds

This section describes the main S&T activities of the INComb project organized according to the work packages presented in the Section 4.1.2, above. All published scientific papers and reports are listed in section 4.1.3.7 (with indication of the respective relevant deliverables in the work packages). In general, all objectives initially defined by INComb have been reached and the final report includes 60 published full scientific papers, 39 reviews and theses and 81 abstracts at scientific meetings where the project results have been presented. The published material is also uploaded via the Commission home page.

4.1.3.1 Work package 2: Modulator release

A first objective resulting in the first deliverable (**D2:1**, for relevant references resulting from the project see publication list in Section 4.2.3.7) was to establish high resolution techniques for measurement of signalling components in the bladder wall. Novel microelectrode techniques were developed, sensitive to the main excitatory transmitters in the bladder wall: the purinergic agonist, ATP and the cholinergic agonist, Acetylcholine. In a parallel effort, a bioassay system was established. In principle compounds released from the urinary bladder were added to another smooth muscle tissue to detect effects on contractility. It was found that the urothelium-containing urinary bladder released a relaxant factor, a urothelium-derived inhibitory factor (UDIF). In a second objective (deliverable **D2:4**) the new techniques were applied to examine the release of transmitters from the urothelium when it was subjected to external physical, biophysical and chemical stresses. The effects of metabolic signalling and transmitters were examined revealing novel feedback mechanisms for sensing bladder filling. New high-throughput methods to measure ATP and acetylcholine release from isolated urothelial cells were developed that allows for effective screening of agents that modulate release of sensory neurotransmitters. In a third objective extensive work was performed to characterize the nature of the UDIF (**D2:2**). The substances released from the urothelium were analyzed using high performance liquid chromatography (HPLC) and the release of different prostaglandins was identified. The effects of these compounds were analyzed in isolated muscle preparations from the bladders to characterize the respective receptors.

Clinical work has shown that botulinum toxin A is effective in suppressing overactive bladder behaviour in patients generally refractory to, or incompliant with, antimuscarinic agents. A further objective of this WP was to study the effects of botulinum toxin on the release of mediators in the bladder wall to better understand the mode of action of the toxin. A first step was to measure the number of suburothelial interstitial cells in biopsies from patients before and after treatment with botulinum toxin-A. Biopsy samples were obtained from patients with stable bladder and those with idiopathic or neurogenic detrusor overactivity. In the detrusor overactivity groups samples, were also taken four and 16 weeks after botulinum toxin-A treatment when clinically-measured detrusor overactivity was reduced. Detrusor overactivity was associated with an increased number of suburothelial interstitial cells, but the number was not reduced by treatment with botulinum toxin-A. A methodology was developed to measure accurately the number of interstitial cells. The implication is that the ability of botulinum toxin to suppress detrusor overactivity is not related to suppression of interstitial cell number (**D2:3**).

This work package also included an examination of possibilities to use microdialysis sampling techniques to detect modulating substances in the bladder wall (**D2:4-5**). The technique involves the insertion of a small catheter into the tissue to collect signalling

substances. Although the technique has interesting possibilities, the approach was not pursued in the current project, since the modulator compounds had a short lifetime and since the catheter most likely would disturb the sensitive micro environment in the bladder wall. Since the high resolution electrode techniques and bioassays provided significant approach the activities were focussed in these techniques.

In summary, this workpackage has developed novel techniques for analyzing signalling components, identified novels stretch and metabolism sensitive feedback signalling and identifying potential substances (prostaglandins) involved in the UDIF signalling.

4.1.3.2 Work package 3: Sensory innervation and TRPV signaling

The urinary bladder has an extensive sensory innervation, which constitutes an integral part of the normal filling sensation. Possibly it also contributes to urgency sensation and detrusor overactivity associated with OAB. The main sensory afferents in the bladder are myelinated Aδ fibres, which are considered to transmit volume and stretch information from the bladder, and unmyelinated C-fibres which transmit noxious sensations. The transient receptor potential vanilloid receptor TRPV1, previously known as the vanilloid receptor, is the original member of the transient receptor potential vanilloid subfamily (TRPV) which includes at present six members. In the urinary bladder of mammals, including man, TRPV1 is largely expressed in sensory fibres, urothelium and interstitial cells. The TRPV1 has recently been identified as an important target for the treatment of OAB. Both capsaicin and resiniferatoxin, which desensitize TRPV1-receptors, have been proven in clinical studies to be effective in treatment of OAB of neuropathic origin.

In a first objective (**D3:1**) the presence and functionality of the TRPV1 receptor was examined in human urothelial cells. The presence of TRPV1 was confirmed and the levels of TRPV1 were not altered by the presence of growth factors in the culture medium. However, when human urothelial cells were cultured in medium supplemented with pro-inflammatory molecules, the expression of TRPV1 more than triplicated. This result also indicates that human urothelial cells respond directly to those pro-inflammatory agents. The functionality of TRPV1 was further assessed and it was observed that TRPV1 responds to capsaicin, heat and pro-inflammatory molecules. It was also concluded that activation of TRPV1 receptors leads to release of ATP by urothelial cells. Altogether, these results demonstrated a possible role of TRPV1 during inflammatory bladder conditions (cystitis).

In a next step the possibilities to treat overactive bladder conditions with small molecule TRPV antagonist(s) was examined (**D3:2**). The effects on bladder dysfunction of a TRPV1 antagonist GRC 6211, a urea derivative, which is absorbable through the intestine were examined. The antagonist caused a dose-dependent reduction in the frequency and amplitude of bladder contractions in spinalized rats which indicates a pivotal role of TRPV1 in neurogenic induced bladder overactivity and offers new forms of treatment to this bladder dysfunction.

A third objective (**D3:3**) was to study the role of neurotrophic factors on the overactive bladder and on the TRPV1 expression in the bladder. It was found that sequestration of the growth factors NGF and BDNF improved referred pain and bladder hyperactivity. Animals were therefore treated with NGF or BDNF. Acute administration of NGF did not affect bladder reflex activity whereas chronic administration caused bladder hyperactivity. For BDNF, acute administration caused bladder hyperreflexia that was not maintained by chronic

administration. These results suggest that BDNF and NGF effects on bladder function depend on the duration of exposure to each neurotrophin. To examine if these growth factors could be used as biomarkers, the urinary levels of NGF and BDNF were determined in patients with overactive bladder syndrome (OAB). It was found that the concentration of NGF and BDNF was very low in the urine of healthy volunteers, irrespective of gender or time of collection. In contrast, in OAB patients urinary NGF and BDNF were significantly higher and decreased after treatment (lifestyle intervention and antimuscarinic drugs). Importantly, urinary BDNF was high in OAB but not in patients with stress urinary incontinence, a confounding pathology, allowing the discrimination between the two groups. Statistical analysis indicated that urinary BDNF is a more sensitive biomarker than NGF.

In a very interesting further project the role of the sympathetic nervous system in the bladder pain syndrome/interstitial cystitis (BPS/IC) was investigated (**D3:4**). In a clinical project, it was found that BPS/IC patients had considerably higher levels of urinary noradrenaline than healthy individuals and an abnormal response of the sympathetic system. Following detrusor injections with botulinum toxin, the levels of urinary noradrenaline significantly decreased, accompanying symptomatic improvement. Based on the clinical results an experimental model was developed to investigate the effects of chronic adrenergic stimulation. Rats received daily subcutaneous injections of phenylephrine, an exogenous agonist for the sympathetic α1-adrenergic receptor. These rats developed bladder hyperactivity and pronounced signs of pain behaviour. Bladder hyperreflexia and pain were accompanied by increased expression of c-Fos in the spinal cord, a marker of sustained sensory barrage in lumbosacral spinal cord. Histological analysis of the bladder showed areas of urothelial erosion and loss of umbrella cells. A significant number of mast cells were present in the lamina propria underlying the eroded urothelium. These results strongly point to an important, and until now, disregarded role of the sympathetic nervous system in the ethiology of BPS/IC.

The relative expression and possible co-localisation of the TRPV variants in different animal models were examined (**D3:5**). The relation between TRPV1 and TRPV4 receptor types was first investigated. Both receptors are present in the bladder where they modulate the organ function. It has been shown that TRPV1 is expressed in urothelial cells and sensory neurons. TRPV4 is present in the urothelium. Whether TRPV4 is expressed in sensory neurons is still controversial. Co-localization of both receptors in small-to-medium sized neurons present in the L6-S1 dorsal root ganglia was demonstrated. Approximately one third of the TRPV1 expressing population also expressed TRPV4. Likewise, one third of the TRPV4 immunoreactive neurons also expressed TRPV1. The extent of colocalization decreased in animals with bladder inflammation. In an animal model of cystitis, the effects of TRPV1 and TRPV4 antagonists, respectively SB366791 (SB) and RN1734 (RN) were studied. These compounds were applied either individually or in combination. Neither antagonist affected bladder function in intact rats. In contrast, administration of either SB or RN resulted in a reduction of the bladder hyperactivity induced by cystitis. Co-administration of SB and RN was also able to reduce bladder hyperreflexia, but this occurred with lower doses of these compounds. These results suggest that TRPV1 and TRPV4 are expressed in different populations of bladder afferents, with little overlap in normal conditions, a condition that disappears totally during inflammation. This is in accordance with the other finding that TRPV1 and TRPV4 antagonists have a synergistic activity, a relevant observation for therapy of bladder dysfunction.

In a final objective the short and long term consequences of botulinum toxin A (BTX-A) injection on human sensory nerves in the bladder were examined (**D3:6**). In one first study,

the effects of Onabot/A injections in the bladder wall on the parasympathetic innervation was examined. The presence of SV2 and SNAP25, respectively the binding site and the specific target of the toxin, was demonstrated in parasympathetic fibres surrounding the postganglionic parasympathetic neurons present in the bladder wall. Twenty four hours after toxin administration, parasympathetic fibres strongly expressed the cleaved form of SNAP25, an indication of the activity of the toxin. These results indicate that the preganglionic parasympathetic fibres in the bladder are a major target for Onabot/A. As these fibres regulate bladder smooth muscle activity, their inactivation by the toxin may constitute a mechanism explaining the strong effects of Onabot/A on bladder contractions. In another study, the expression of the cleaved form of SNAP25 (cSNAP25), as a marker of toxin spread and activity in the bladder, was examined. The influence on the spread in the bladder wall of injected Onabot/A by dosage of toxin or volume was characterized. Two routes of toxin administration, injection in the bladder wall and instillation, were applied. Results obtained in this study indicate that instillation of Onabot/A is not effective. In contrast, detrusor injection with Onabot/A resulted in a high expression of cSNAP25, mainly in cholinergic fibres, the main target of the toxin. This effect was more evident when higher volumes are injected. The findings suggest that the injected volume is a key factor in the spread and action of Onabot/A in the bladder, and this may be relevant to improve the clinical effect of Onabot/A. The effects of the injection of botulinum toxin in the prostate were investigated as a novel treatment for benign prostatic enlargement caused by benign prostatic hyperplasia. The aim was to investigate the expression of apoptosis-regulating proteins in the rat prostate following botulinum toxin intraprostatic injection. The results obtained indicate that botulinum toxin activates apoptotic pathways in the rat prostate through a mechanism that involves sympathetic outflow impairment. In a clinical project, the effect of bladder injection in the trigonal area with botulinum toxin for the treatment of bladder pain syndrome/interstitial cystitis (BPS/IC) was investigated. Patients that received up to four injections with intervals of 9 to 12 months between injections were examined. It was concluded that the treatment remained effective and without relevant side effects.

In summary, this work package reached all of its objectives and has presented novel data on the distribution of the TRPV receptors, their effects and their modulation by growth factors. The studies on the sympathethic nervous system in BPS/IC has opened a new area of research that may contribute to the understanding of the pathophysiology of the disease and give rise to the development of new more effective therapies for the treatment of this still incurable disease. Important achievements relate e.g. to the approaches on biomarkers, the role of sympathetic system in bladder pain, the regulation if TRPV receptor expression in disease, and the characterization of Botox distribution in the bladder wall.

4.1.3.3 Work package 4: Interstitial cell function in the bladder

The interstitial cells of Cajal (ICC) are named after the Nobel prize laureate Santiago Ramón y Cajal, who more than 100 years ago described a network of these cells in the gut. It is now established that these cells have important roles in the gastrointestinal tract in mediating peristaltic contractile activity and transmitting signals from nerves to smooth muscle. In the last few years, cells resembling the ICC have been found in tissues of the lower urinary tract, in particular the bladder. While the role of interstitial cells (IC) in the bladder is not well understood, it has been established that they are located in the bladder wall below the urothelium in the lamina propria, in the detrusor where they run in parallel with the smooth muscle bundles and in the spaces between these bundles. Bladder IC have been identified by e.g. labelling with antibodies to the *c-kit* receptor, a tyrosine kinase receptor encoded by the

proto-oncogene *cKit.* The IC have a branched morphology and may form contacts with smooth muscle and intramural nerves. Imatinib mesylate (also known as Glivec) which blocks the tyrosine kinase activity of c-kit has been shown to increase the compliance of the bladder in guinea-pigs in vivo which might suggest that the ICC have a modulatory role on bladder tone. Several important questions remain to be answered: i) are there different subpopulations of IC in the normal urinary bladder and does the IC morphology and proportional size of subpopulations change in OAB?; ii) what are the cellular properties of IC?; iii) which receptors are expressed and functional in IC?; iv) how do IC communicate with smooth muscle and nerve components of the bladder wall?; and v) can the IC be a target for pharmacological therapy.

A first objective was to study the distribution and sub-populations of interstitial cells (IC) in the normal human bladder. This was examined using the established IC markers, anti-c-Kit and anti-vimentin, and confocal microscopy (***D4:1***). A sub-population of c-Kit$^+$ or vimentin$^+$ IC was located in the *lamina propria* mucosal layer, between the urothelium and the *detrusor muscularis*. These *lamina propria* IC had a stellate morphology and formed a network, making associations with cholinergic nerves (labelled with anti-vAChT). Within the detrusor, c-Kit$^+$ or vimentin$^+$ IC were elongated, bipolar cells with several lateral branches which were positioned axially on the boundary of the smooth muscle bundles (stained with phalloidin). These IC did not form networks but were also found close to intramural nerves. Transmission electron microscopy was used to examine the ultrastructural profile of IC in the human bladder and demonstrated that they possessed characteristics typical of those published for IC in other tissues. Human bladder IC were distinct from smooth muscle cells in the absence of dense bodies, dense bands and thick filaments but contained mitochondria, a discontinuous basal lamina, free ribosomes, numerous vesicles, Golgi and a well developed but not dilated rER. A new IC marker in human bladder (platelet-derived-growth-factor-receptor-alpha, PDGFRα) was discovered and provided novel means of labelling IC populations. This is a significant advance for the field as c-Kit antibodies are notoriously difficult to use in the laboratory.

The distribution of suburothelial interstitial cells from human biopsy samples using the immunohistochemical markers, vimentin, connexin43 and c-Kit. There was a band of strongly vimentin positive cells immediately below the urothelium, with cell bodies and long projections parallel to the basal lamina. Cx43 immunolabelling was extensively distributed in the suburothelial layer, coinciding with the layer of vimentin positive cells, and somewhat offset towards the detrusor layer, possibly suggesting that gap junctions projected somewhat away from the urothelium. A sub-population of vimentin- and Cx43-positively labelled cells also labelled with c-Kit. One objective of the study was to determine the relative merits of the different labels to assess quantitatively the distribution of these cells.

A semi-quantitative method to equate the change of label intensity with an increase of cell number was described. The distribution and number of cells was compared in samples obtained from patients with urodynamically-stable bladders and those with neurogenic-associated and idiopathic detrusor overactivity. This was to test the hypothesis that the number of suburothelial interstitial cells was increased in bladders of patients with detrusor overactivity. The other hypothesis to be tested was if treatment of patients with botulinum toxin, to reduce detrusor overactivity, decreased also the number of suburothelial interstitial cells.

In a morphological study it was shown that suburothelial interstitial cell number was increased in samples form neuropathic and idiopathic overactive bladders when using vimentin and Cx43 labelling. Evaluation of data obtained with c-Kit did not show an increase of cell number. The data were qualitatively similar to those observed in animal preparations with neuropathic detrusor overactivity. These data suggest a subset of the suburothelial interstitial cell population is increased in human bladder overactivity. Treatment of patients with botulinum toxin that generated a significant reduction of overactive detrusor function was not associated with a reduction of Cx43-labelled suburothelial interstitial cell number. The data indicate a specific population of suburothelial interstitial cells is increased during development of the overactive bladder in patients

A second objective was to investigate the changes in the distribution and quantities of IC in the overactive human bladder **(D4:2).** The distribution and quantities of IC in OAB tissues were compared with data from the normal bladder. As with animal models the data indicate that the distribution of IC is altered in the overactive human bladder. IC in the lamina propria layer (IC-LP) appear to be increased in number. Of particular note is the finding that in tissue samples from botulinum treated bladders, c-Kit-labelled IC-LP numbers do not change, in spite of the fact that patients experience relief from OAB symptoms. Biopsies were examined from 14 patients with OAB, before and four weeks after BTX-A injection. Quantitative immunoflourescence was peformed using commercially available antibodies to c-Kit and Mast Cell Tryptase (MCT). No significant changes in the number of c-kit positive cells were identified from before to after BTX-A injection. A significant decrease in the number of MCT positive cells was seen after BTX-A in the NDO population and a similar trend was observed in the IDO population although this did not reach significance. This finding may reflect an anti-inflammatory effect of BTX-A treatment.

The use of the novel marker PDGFRα has presented the possibility to examine populations of IC in OAB which are not KIT^+. In animal models of OAB, IC may undergo phenotypic changes. This is particularly interesting since human OAB samples indicate that there may be more $PDGFR\alpha^+$ cells in human neurogenic OAB than KIT^+ cells.

Several participants within the consortium have worked together to investigate IC expression within several complementary animal models of bladder overactivity (**D4:3**). Confocal imaging of bladder samples which had been labelled with anti-vimentin revealed that populations of detrusor IC and lamina propria IC were reduced in number after spinal cord injury (SCI) in rodents. Bladders from spinal cord injury (SCI) rats, as well as being hypertrophied were more compliant than those from sham-operated animals. When strips were prepared for isometric tension recordings, those from SCI bladders relaxed at a greater rate compared to sham-operated and non-operated controls. These data suggest bladders from SCI animals had become decompensated. Spontaneous contractions of longitudinal detrusor strips were longer and less frequent from SCI animals (0.05 Hz) compared to sham-operated (0.12 Hz) or non-operated controls (0.10 Hz) and of greater amplitude (when differential wall stiffness was accounted for) than in sham-operated animals. Thus, spontaneous contractions revealed a partial OAB phenotype. Taken together, the findings of this study show that in SCI rodents, reduction in the number of IC, disruption in the integrity of IC 3D arrangements in addition to disruption of innervation patterns, is associated with a decompensated and partial OAB phenotype. Interestingly, altered contractile activity is also found in newborn and neonatal urinary bladders. A mouse model for bladder growth was characterized and the interaction between signalling pathways regulating contractions. It is the first study determining these signalling pathways simultaneously in the hypertrophying bladders. It was

found that the sustained component of the contraction was increased in the hypertrophying bladders and that PKC and RhoA signalling was unchanged. Interestingly, it was also found that strong stimulation of PKC with PDBu resulted in a prominent contraction in the hypertrophying mouse bladders. This contraction was inhibited by Blebbistatin (an inhibitor of non-muscle myosin). Cells with expression of non-muscle myosin were identified in the bladder wall. These data suggest that bladder growth can result in development of new contractile components in the wall, possibly contributing to wall stiffness and regulated by different pathways compared to the bladder smooth muscle.

Interstitial cells have close contacts with the intramural nerves in the urinary bladder and to examine the role of the nervous input in IC cell structure and function, NComb examined the distribution of IC and contractility in the denervated rodent bladders (**D4:4**). The denervated bladders had undergone hypertrophy and had significantly greater mass than sham operated controls. Confocal imaging of tissues labelled with vimentin antibodies revealed that IC in the *lamina propria* had similar numbers and morphological arrangement as the sham operated controls, however the detrusor IC were significantly reduced with disruptions to their morphological architecture. These observations were supported by electron microscopy ultrastructural studies. These findings indicate that OAB in the denervated rodent bladder model is associated with disruption of the detrusor IC population.

The suburothelial interstitial cells (ICs) in the bladder mucosa are proposed to play a key role in the transduction of bladder sensation from the urothelium to afferent activation, as well as influencing directly detrusor contractile function. This is because ICs are excited by urothelial transmitters, their immediate metabolites or other endogenous agents such as H^+. We therefore examined the cellular physiology of isolated sub-urothelial IC to evaluate their sensitivity to different agonists and ability to propagate activity after stimulation (**D4:5**). It was demonstrated that suburothelial cells generate large inward currents when exposed to ATP and P2Y agonists (ADP, UTP, UDP), in addition P2Y agonists increase the mucosa-related spontaneous contractions, implying a paracrine effect between the two layers. Furthermore, reduction of pH below 6.5 also generates large IC responses, whilst nitric oxide (also released from urothelium) reduces ATP responses. In all cases inward currents are preceded by intracellular Ca^{2+} transients. Capsaicin, also reduced inward current, but not the Ca^{2+} transient, implying it may block directly the current and suggest also that reduction of pH does not act through a $TRPV_1$ receptor. Of interest also, is that IC responses are augmented when two cells are in close contact, but without forming gap junction complexes. This will happen particularly when the number of ICs is increased, i.e. in overactive bladders, and may contribute to the upregulation of spontaneous contractile activity. This augmentation was reduced by the c-Kit receptor antagonists Glivec, implying that physical cell contacts increases the cellular pathways responsible for the agonist induced Ca^{2+}-transient and inward current.

This work was followed by a more in depth characterisation of the effect of P2Y agonists on suburothelial Ca^{2+}-wave and electrical activity and its influence over detrusor function. These results showed that ADP, UTP and UDP – which depress detrusor function *per se* – increase spontaneous contractions through augmenting signalling between suburothelium and detrusor

The cellular physiology of detrusor IC as individual cells and within bladder sheets was investigated. Patch-clamp experiments have shown that detrusor IC possess novel K^+ channels (KCNQ) channels which contribute to the control of cellular excitability. The potassium currents in detrusor IC were found to be sensitive to typical environmental factors

such as stretch (via hypo-osmolar solution) and H^+. Pharmacological evaluation suggests that a component of the whole cell potassium current may be carried by 2-pore domain potassium channels such as TREK and TASK; moreover immunohistochemical staining shows that detrusor IC express TASK and TREK ion channels. Detrusor IC fire spontaneous transient outward currents (STOCs) via K^+ channels (BK type) as demonstrated by their sensitivity to the BK channel blockers. Spontaneous release of Ca^{2+}-from intracellular ryanodine-sensitive stores activates the BK channels; stores are replenished by Ca^{2+}-influx via L-type Ca^{2+}-channels and other Ca^{2+}-entry pathways. The predominance of hyperpolarizing signals argues against detrusor IC acting as pacemakers in the bladder wall and may indicate a role in inhibiting the activity of neighbouring detrusor smooth muscle during bladder filling when the bladder is relaxed. Application of the major excitatory neurotransmitters, carbachol (acetylcholine analogue) and ATP had differing effects on detrusor IC. Carbachol increases STOC activity by transiently increasing their amplitude and duration whereas ATP evokes non-selective cation currents in a sub-group of cells tested. Transmission electron microscopy of guinea-pig bladder IC demonstrated that the cells contained typical secretory apparatus such as cytoplasmic vesicles, abundant rER and Golgi complexes. Application of carbachol to enzymatically dispersed detrusor IC increased the number of vesicles, indicative of a paracrine function. The physiological activity and Ca^{2+}-signalling patterns of bladder IC were examined demonstrating distinctively different signalling patterns in IC at different locations in the bladder It was also shown the that blockade of KIT and PDGFR signalling (acting on ICs) in bladder tissue does not prevent spontaneous activity of the bladders and therefore does not support a role for IC in the origin of this activity. Overall, this work suggests that bladder myogenic spontaneous activity may be modulated in different ways by IC in the different compartments of the bladders wall.

Different external signalling pathways affecting IC function and bladder contractility were examined (**D4:7**). In a rodent model with spinal cord transection (SCT) an increased spontaneous contractile activity in the isolated bladder is observed, reminiscent of an overactive human bladder syndrome with similar damage to the central nervous system. An increase of interstitial cell number in the suburothelial space between urothelium and detrusor smooth muscle occurs in SCT bladders and these cells elicit excitatory responses to pyrimidines and purines such as ATP, ADP and UTP. The hypothesis that these agents underlie increased spontaneous activity was tested. SCT bladders were associated with regular spontaneous contractions; ADP, UTP and UDP augmented the amplitude but not their frequency. With strips from such bladders, a P2Y6-selective agonist (PSB0474) exerted similar effects. Fluorescence imaging of bladder sheets showed that ADP or UTP increased the conduction velocity of Ca^{2+} waves that were confined to regions of the bladder wall with an intact mucosa. Analysis of wave propagation showed that the suburothelial space exhibited properties of an electrical syncytium. These experiments are consistent with the hypothesis that P2Y-receptor agonists increase spontaneous contractile activity by augmenting functional activity of a cell syncytium in the suburothelial space. The spontaneous activity of mucosal, detrusor and intact (i.e. detrusor plus mucosa) strips were compared to test the hypothesis that spontaneous contractions are of different origin. The mean spontaneous activity was greatest in intact strips compared to detrusor and mucosa and the spontaneous contractile activity in different regions had different sensitivity to agonists acting on purino-, adeno- and TRPV-receptors. The results suggest that spontaneous contractile activity in the urinary bladder can have different origin and that the fncdtional pacemaker cells can have different sensitivity to physical, metaboloc and pharmacological interventions

In summary, this workpackage was the largest in the INComb project and has involved extensive work in many laboratories and several collaborative interactions. All objectives were reached and all deliverables are presented together with a large number of papers, manuscripts reviews and abstracts. Several novel properties and functions of the bladder ICs have been reported as presented above. Novel markers for ICs have been identified (PDGPRα), receptor properties characterized (P2Y receptors) and the subpopulations of the ICs described. These may provide promising new ways to modulate bladder activity and properties in OAB via more selective IC targeting.

4.1.3.4 Work package 5: Myogenic mechanisms and inter-cellular communication in the bladder

The different cellular components in the bladder wall including smooth muscle, urothelium, interstitial cells and nerves, form a complex integrated signalling network. During bladder filling and micturition, the relaxation and contraction of smooth muscle is coordinated by interaction between these different cells. This work package focussed on the causes of spontaneous contractile activity and on the properties of cellular communication. Gap junctions are plaque-like regions of the cell surface where transmembrane hemichannels ("connexons") of adjacent cells join. Each connexon is formed by a hexameric cluster of structural proteins belonging to the family of connexins. By allowing intercellular transmission of second messengers, ions and small metabolites, gap junction communication creates a functional network of electrically or metabolically coupled cells.

As first step the distribution and subtypes of connexins (Cx) in the bladder wall of stable and overactive bladders was analyzed in animal and human tissues (**D5:1**). Rats urinary bladder was obtained from a model of spinal cord injury to generate an overactive bladder phenotype. Human tissue was obtained from patients with stable or overactive (idiopathic or neuropathic) bladders. In addition samples were also obtained from overactive bladder patients 4 and 16 weeks after botulinum toxin treatment to suppress bladder overactivity. An improved technique for quantitative estimation iof the Cx43 density was developed In the suburothelium Cx43 is the predominant subtype and its distribution was examined in these cohorts. In the overactive bladder Cx43 was upregulated with an increase of Cx43 labelling per unit area. These data were in accordance with those from rats where there was also a large upregulation of Cx43 labelling in the suburothelium. Cx43 immunolabelling was extensively distributed in the suburothelium layer, coinciding with the layer of vimentin positive cells, and sometimes slightly offset towards the detrusor layer, suggesting that gap junctions, located on the cell filaments, projected away from the urothelium, i.e. the boundary of the Cx43 positive band was more sharply defined on the urothelium-facing side than on the submucosal side where the labelling density tended to decrease progressively with depth. Cx45 immunolabelling was not detectable in the suburothelium.

Bladder tissue contains several different connexins and the occurrence and distribution of all known 21 human and 20 murine connexin isoforms was examined using RT-PCR with isoform-specific primers. The presence was demonstrated of many more different connexin isoforms in the bladder than previously known and distinct differences in isoform expression patterns between mouse and man were observed. Using combined laser capture microdissection/PCR approach the isoform transcripts could be assigned to different compartments of the bladder (urothelium, suburothelial connective tissue and detrusor smooth muscleIn. both man and mouse, the urothelium expressed the greatest spectrum of isoforms (10 isoforms in mouse; 11 isoforms in humans). In suburothelial connective tissue, mice

express 9 isoforms while humans only 5. The detrusor (smooth muscle) of mice also displayed a wider range of isoforms than humans (mouse: 8 vs. human: 5). Changes of connexin31, one of the strongly expressed connexins in urothelium of both species, was further investigated in a mouse model with experimental bladder outlet obstruction. Connexin31 could be specifically localized in basal and intermediate urothelial cells with most prominent staining at apical cell membranes. The same localization was found in human bladder samples. Urinary outlet obstruction and hypertrophy in mice was associated with a significant rise in connexin31 transcription.

In an associated objective the effects of different connexin modulators were examined in models of bladder overactivity were examined (**D5:2**). Studies were performed on adult female rats five weeks following spinal cord injury (SCI) to obtain a state with overactivity resembling OAB. The connexin-mimetic peptide Gap27, postulated to reduce gap junction conductance, stimulated an increase in basal tone and amplitude of spontaneous contractions. The gap junction uncoupler carbenoxolone did not affect either basal tone or the amplitude of spontaneous contractions in SCI or sham-operated strips, but produced a concentration-dependent increase in these measures in un-operated controls. In a separate study carbenoxolone also increased the frequency of spontaneous contractions in detrusor strips from un-operated guinea pigs. In order to address the observation of a paradoxical stimulation of spontaneous contractions by gap junction uncouplers, oil gap experiments assessed junctional resistance in un-operated rat detrusor strips. Further experiments are required to understand whether gap junction uncouplers may have an effect comparable to a desensitizing agonist (i.e. first decreasing, then subsequently increasing gap junctional resistance). In another model of altered bladder contractile pattern (and possibly OAB), the bladder of newborn mice, the gap junction inhibitor 18-β-glycyrrhetinic acid was examined. The compound inhibited agonist induced phasic activity phasic in adult and newborn bladders in a similar manner. The phasic activity of the newborn was not specifically affected by the gap junction inhibitor. In a subsequent study it was found that the phasic activity of the newborn bladder was modulated by $NiCl_2$ suggesting that it involves action of T-type Ca^{2+} channels.

The inter-relationship between the urothelium, suburothelium and detrusor muscle layers was investigated to determine how signals propagate between these different layers and where these signals originate from (**D5:3**). The aim was to understand the cellular pathways whereby spontaneous contractions develop and propagate in the normal and overactive bladder. These studies utilised rat bladder subjected to spinal cord injury to generate an overactive phenotype and a sham group. Experiments used optical imaging whereby the bladder was loaded with Ca^{2+} and membrane potential-sensitive fluorochromes and separate regions of the bladder surface or cross-section were imaged to record Ca^{2+} and membrane potential transients. With normal bladders there were several origins of activity, each generated a localised response and correlated with frequent small contractions. With overactive bladders there were one or two foci that propagated across the bladder wall and these correlated with less frequent but large bladder contractions. Partial removal of the mucosa (urothelium, suburothelium) demonstrated that it was required for the large spontaneous contractions in the overactive bladders. Imaging cross-sections of the bladder wall showed that foci of activity originated in the suburothelium and propagated either towards the urothelium or towards the detrusor layer. When propagating to the detrusor, signals were often significantly delayed at the interface between these two regions but always ultimately occurred. Signals propagated from the suburothelium foci at a rate that suggested cell-to-cell electrical connectivity in the suburothelium – signals were predicted to propagate at a rate suggested by cable theory and demonstrates that this layer acts as a funtional syncitium.

Additional experiments investigated the presence of spontaneous electrical activity and Ca^{2+} transients in isolated human detrusor myocytes from stable and overactive bladders. Transients were more frequent in overactive bladder myocytes and were reduced in number by L-type and T-type Ca^{2+} channel blockers, as well as an inhibitor of intracellular Ca^{2+} stores, CPA. These data provide evidence that in human detrusor a myogenic component of overactive bladder behaviour is also present.

The cell signalling aspects in the contractile activation of the detrusor muscle was analyzed in a transgenic mouse with conditional smooth muscle specific knockdown of Rac1. Rac1 is a small G protein of unknown function, but potentially involved on cell signalling to the muscle cytoskeleton. It was shown that knock down of Rac1 or application of specific Rac1 antagonists inhibited the receptor induced activation in smooth muscle of bladder and vascular smooth muscle, providing evidence for a new and potentially targetable pathway in the activation of smooth muscle. The metabolic signalling and the effects of an AMPK-activator (AICAR) were examined. AMP-activated kinase is a metabolic sensor affecting several muscle functions and AICAR has been proposed to be used to promote glucose uptake in skeletal muscle. It was found to be expressed in arteries and bladder with a more prominent AICAR effect in arteries, resulting in relaxation due to a PKC dependent pathway.

Organ culture systems (**D5:3**) were developed during the first part of the INComb project to enable long term *in vitro* studies of isolated tissue. New techniques to maintain human and animal bladder tissue with intact contractility in organ culture conditions were developed. The effects of imatinib mesylate (Glivec) on IC were analyzed.

Transurethral microwave thermotherapy (TUMT) is used clinically to treat human benign prostatic hyperplasia symptoms. Apoptosis and necrosis of cells can be involved, but modulation of neuronal components of tissue including the sensory neurons of the posterior urethra can also contribute to the beneficial effects. We hypothesized that local heat treatment can affect sensory nervous signalling in the bladder wall, possibly via TRPV receptors a mechanisms similar to capsaicin which can affect OAB symptoms when instilled in the bladder. Potentially thermal treatment can be an interesting approach although it was realized that this task was explorative in its nature and a positive outcome with clinical applicability could not be guaranteed.

We addressed the effects of mild heating on bladder contractility. Heating was applied on isolated urinary bladder tissue from 37 °C to 42 °C for a prolonged period (15 minutes) or repeated short exposures (1-minute cycles). It was found that this treatment altered the spontaneous contractility of mucosa-intact bladder strips. During 15 minutes of exposure to 41±1°C rabbit mucosa-intact preparations exhibited significantly reduced spontaneous contraction amplitude compared to control preparations maintained at 37±1°C. Post-heating, the amplitude of spontaneous contractions returned to control values within 30 minutes. During this recovery period there was a reduction in the frequency of contractions in the previously heated preparations in comparison to the control preparations. Heating did not produce notable changes in bladder wall morphology (urothelium width, sub-urothelium cellular density or the muscle content) of heated (41°C) strips, compared to controls (37°C). There was no evidence of motor nerve block. Experiments where heating was performed in the presence of the TRPV1 antagonist capsazepine suggested that the suppression of spontaneous contractions on heating may be mediated by TRPV1 receptors

In a separate study the effects of electromagnetic field (EMF)-induced hyperthermia was examined in *in-vitro* studies on isolated murine bladders. For this purpose, bladders from normal mice were dissected and exposed for about 1 h to EMF resulting in a temperature of 41°C. Histological analyses revealed morphological damages – especially of smooth muscle and urothelium - in both control and EMF-treated samples, that, however, was more prominent after heat-exposure. Analyses showed that hyperthermia resulted in significantly lower transcript numbers of all genes investigated after end of microwave treatment which can be due to a true down-regulation of gene expression or attributed to a degradation of RNA and/or apoptosis of cells at high temperatures. At least some of the detrimental effects may be transient in the bladder. Whether the tissue is able to completely convalesce from hyperthermia-related damage and what are the consequences for bladder functions or under disease conditions remains to be clarified. The results thus suggest that mild heat treatments might affect the contractility of bladder tissue, but the mode of introducing the heat and the potentially negative effects of electromagnetic field techniques remain to be investigated before translating these results to the *in vivo* situation.

In summary, this work package reached its objectives, and reported on several aspects of muscle contraction and intercellular communication in the bladder wall. A new signalling pathway with a potential role in receptor activation was demonstrated and that metabolic signalling is a potentially important aspect in regulating bladder tone. The work on connexins has revealed expression of yet uncharacterized isoforms and identified expression in the human bladder. Heat treatment provides a novel non pharmacological mode of affecting contractility and the preliminary results suggest a potential beneficial effect via action on TRPV receptors. However the initial studies of electromagnetic wave treatment, suggesting potential negative effects, introduces some caution before translating the technique to the human situation, without careful consideration of dose effects.

4.1.3.5 Work package 6: Receptor signalling in different components of the bladder

The receptor subtypes and their signalling mechanisms in the urothelium and their role in the release of mediators affecting afferent nerves and/or smooth muscle cell tone were examined according to a first objective (**D6:1**). Commercially available receptor antibodies were evaluated and new radioligands developed. In line with emerging work on several other receptor systems, it was found that most available muscarinic receptor or adrenoceptor subtype antibodies lacked adequate selectivity for their target. As the main adrenoceptor interest became β_3-adrenoceptors a more in depth evaluation of antibodies against human and rodent β_3-adrenoceptors was performed. This resulted in the successful identification of selective antibodies for both human and rodent receptors It was found that the radioligand [^{125}I]-iodocyanopindolol can label β_3-adrenoceptors in rat bladder but only with an extremely high non-specific binding yielding considerable uncertainty in the interpretation of the results. Therefore, the generation and validation of a more suitable β_3-adrenoceptor radioligand based on the selective antagonist L-748,337 was initiated. While this ligand still was found to be not optimal, it outperformed previously available radioligands for the labeling of β_3-adrenoceptors.

To establish and validate the best model system for the functional studies, the quantitative expression pattern of > 40 GPCRs in various types of human urothelial cells, i.e. freshly prepared urothelium, two immortalized urothelial cell lines (UROtsa and TERT-NHUC) and one urothelium-derived cancer cell line (J82) was examied. These experiments demonstrated

that a major fraction of GPCRs is down-regulated in all three cell lines as compared to fresh urothelium, some even to non-detectable levels. Expression of a very small number of receptors was largely maintained in some of the cell lines; among the three cell lines, the UROtsa cells exhibited the most similar expression profile as compared to freshly isolated urothelium. Based upon these screening results, further efforts were focused on three GPCR families, i.e. muscarinic, bradykinin and protease-activated receptors (PARs), as expressed in UROtsa cells.

Initial studies on muscarinic receptors in UROtsa cells detected mRNA for all 5 subtypes of muscarinic receptors (mostly M_2 and M_3) along with that of carnitine acetyltransferase and choline acetyltransferase. These receptors coupled to inhibition of cAMP accumulation and to stimulation of inositol phosphate formation, leading to enhanced cell proliferation, the latter response involving activation of ERK and PI3/kinase. In follow-up of this carbachol-induced intracellular Ca^{2+}-elevations but nevertheless acetylcholine and NO release were very small or undetectable. On the other hand, a robust stretch-induced ATP release from UROtsa was detected. Activation of gap junction hemichannels was observed, potentially a novel mechanism responsible for stretch-induced ATP release in urothelial cells which could be modulated by GPCR agonists, such as bradykinin or thrombin.

The bradykinin receptor system was investigated in UROtsa cells. Both B1 and B2 bradykinin receptor mRNA and concentration-dependent B2-mediated Ca^{2+}-elevation, ERK phosphorylation and ATP release, was detected. However neither acetylcholine nor NO release in quantifiable amounts was observed. Most interestingly NGF mRNA and protein and TRPV1 mRNA were detected. Bradykinin stimulated NGF release from UROtsa cells in a phospholipase C-, protein kinase C- and ERK-dependent manner. Bradykinin receptor function and the underlying signal transduction in detrusor smooth muscle was examined in isolated rat bladder strips. Bradykinin caused weaker contraction of rat urinary bladder than muscarinic stimulation. A phospholipase C inhibitor did not significantly affect the bradykinin response, whereas a phospholipase D inhibitor caused a weak but significant inhibition. A cytosolic phospholipase A_2 inhibitor, a cyclooxygenase inhibitor and an L-type Ca^{2+}-channel blocker caused strong inhibition of the bradykinin response, whereas only a small but significant inhibition was seen with an inhibitor of receptor-operated Ca^{2+}-channels. Several protein kinase C inhibitors did not yield an unequivocal picture, whereas a Rho kinase inhibitor caused a strong and concentration-dependent inhibition of the bradykinin response.

UROtsa cells expressed mRNA for PAR subtypes 1-4 and in response to various agonists (thrombin, trypsin, PAR-1 and PAR-2 agonistic peptides) raised intracellular Ca^{2+}-concentrations in a phospholipase-dependent manner. It was reported for the first time, that PAR-1 and PAR-2 agonists enhance stretch-induced urothelial ATP release by increasing intracellular Ca^{2+} levels, activating PKC, Rho GTPases and gap junction hemichannels. Moreover PAR-1 and PAR-2 activation led to increased urothelial NGF release, indicating that these receptors may provide novel target to prevent various mediator release from urothelium.

Since β_3-adrenoceptors are an important emerging drug target in incontinence treatment further studies were initiated. The β_1-selective antagonist nebivolol had been proposed to also be a β_3-adrenoceptor agonist. However, it was found that nebivolol had only low affinity at β_3-adrenoceptors; it did not display agonism for cAMP elevation with cloned human β_3-adrenoceptors or rat bladder β-adrenoceptors and also did not cause relaxation of isolated rat bladder strips. Two selective β_3-adrenoceptor agonists undergoing clinical development were

examined mirabegron and FK 4664. these were studied with cloned human β_3-adrenoceptors in radioligand binding and cAMP experiments and in relaxation experiments with isolated rat bladder strips. Effects of KUC-7322, the active metabolite of ritabegron, were studied for relaxation of isolated human bladder strips.

To study the interaction between receptor systems, particularly between subtypes of muscarinic and adrenergic receptors, in the urinary bladder, several cell lines expressing M_2 or M_3 muscarinic or β_3-adrenergic receptors were generated, (**D6:2**). Unfortunately, only very few of the many cell lines which have been made co-expressed β_3-adrenergic receptors as assessed functionally by isoprenaline-induced cAMP accumulation. The co-expressing cell lines failed to exhibit alterations of muscarinic receptor signalling at baseline as compared to those expressing only M_2 or M_3 receptors, and also failed to demonstrate such alterations upon exposure to isoprenaline. INComb scientists therefore chose to exploring the receptor interactions in rat bladder strips in vitro. Using a novel and highly M_2-selective antagonist as a masking agent it was possible to generate M_3-selective stimulation and using an M_3-sparing agonist an M_2-selective stimulation was achieved in the bladder. The data show that both muscarinic subtypes contribute to the attenuation of β-adrenergic relaxation and that phospholipase C, but not protein kinase C, is involved in such attenuation. In a second approach it was tested how pre-treatment with β-adrenergic agonists affects contractile responses in rat bladder strips in vitro. This work compared a general β-agonist (isoprenaline), a β_2-agonist (fenoterol), and a β_3-agonist (mirabegron). All three agonists attenuated contractile responses to the muscarinic agonist carbachol and also receptor-independent contractions to KCl after 6 h pre-treatment followed by thorough washout, and such attenuation was independent of the presence of urothelium.

Changes in expression and/or function of α_1- and β-adrenoceptor subtypes as a cause for bladder dysfunction were explored in a rat bladder outlet obstruction (BOO) model induced in rats by partial urethral ligature with subsequent in vitro testing of isolated bladder strips obtained 7 days after BOO induction (**D6:3**). Receptor-independent contraction or relaxation did not differ between BOO and sham rats. The α_1-agonists methoxamine and A-61,603 caused only weak contraction without major differences between groups. Against KCl-induced tone, the β-adrenoceptor agonists noradrenaline and isoprenaline caused similar relaxation in BOO and sham rats, whereas relaxation in response to the β_3-selective BRL 37,344 was attenuated. Against passive tension, noradrenaline induced relaxation in sham and control rats; in contrast, noradrenaline induced contraction at low concentrations and relaxation at high concentrations in BOO rats. The contraction component was abolished by the α_1-antagonist prazosin. The mRNA expression of α_{1D}-adrenoceptors was increased in BOO, whereas none of the other receptor mRNAs were up-regulated. In a rat BOO model, weak contraction responses to α_1-agonists and relaxation responses to β-agonists are not altered to a major extent. Nevertheless, relaxation responses to the endogenous agonist noradrenaline are turned into α_1-adrenoceptor-mediated contraction responses in BOO, possibly due to an up-regulation of α_{1D}-adrenoceptors.

Desensitization can limit responses to agonist treatment, a situation with potential applicability to overactive bladder treatment with β_3-adrenoceptor agonists. In experiments with cloned human β_3-adrenoceptors it was found that such agonist-induced desensitization occurs in some (HEK) but not other cell types (CHO). Characterizing the agonist-induced desensitization in HEK cells it was observed that this occurs not at the level of β-adrenoceptor down-regulation or of regulation of G-protein α-subunits but apparently rather at the level of adenylyl cyclase. The above cell type differences in susceptibility to desensitization

demonstrate the need for studies in target tissue. Therefore, studies with isolated rat bladder using general and subtype-selective β-adrenoceptor agonists for pre-treatment were performed. In rat bladder, desensitization was more pronounced for the β_2- than the β_3-adrenoceptor component of relaxation, and within the latter occurred only for some but not for other agonists; in contrast to the findings in HEK cells, this apparently did not occur at the level of adenylyl cyclase. Using the same conditions of pre-treatment with subtype-selective β-adrenoceptor agonists we also studied heterologous desensitization of contractile responses, which was detected for muscarinic but not receptor-independent contractile stimuli.

In a series of consecutive patients undergoing bladder surgery the possible associations of potency and efficacy of a β-adrenoceptor agonist with gender or age were explored, but did not detect major effects with the given sample size.

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid involved in several biological functions. Recent studies have suggested that S1P can regulate detrusor tone and can interact with muscarinic signalling in the bladder wall. The role of sphingosine-1-phosphate (S1P) and sphingosine kinase in various receptor systems in the bladder and the role of S1P signalling in normal bladder activity and OAB. were examined (**D6:4**). At the mRNA level, all five S1P receptor subtypes and both isoforms of sphingosine kinase were detected in human urothelium; moreover, in contrast to most other receptors, expression of the S1P receptor subtypes was largely maintained or even increased in urothelium-derived non-malignant and cancer cell lines. Of note, the S1P5 receptors, which otherwise exhibits a very restricted expression pattern was also highly expressed in human urothelium. The expression of S1P5 receptor mRNA in the urothelium but not smooth muscle was also confirmed in the rat bladder. S1P receptor subtype presence and function was examined in rat bladder smooth muscle cells. In contrast to human urothelium, rat bladder smooth muscle expressed only S1P1, S1P2 and S1P3 receptor mRNA. Testing a range of subtype-selective agonists it was found that S1P2-mediated elevations of intracellular Ca^{2+} and cAMP accumulation, with both responses being pertussis toxin-sensitive. On the other hand, inhibition of endogenous S1P formation did not affect contractility of multicellular preparations, i.e. isolated rat bladder strips.

Based upon a reported role of S1P in cellular growth responses and hypertrophy InComb participants have explored the role of endogenous S1P in rat bladder under growth-promoting conditions (**D6:5**) employing two paradigms, i.e. bladder strips cultured in the presence of serum and bladder strips isolated from the hypertrophied bladder obtained from BOO rats. Inhibition of S1P formation attenuated contractile responses to a muscarinic agonist under both conditions. This was accompanied by an increased S1P4 receptor mRNA expression under both conditions, whereas expression of other S1P receptor subtypes or isoforms was not altered (full paper in preparation).

In summary, this work package has reached its objectives and generated a significant number of high level papers and reviews bridging over a broad range of problems in the research field. Novel clinically relevant information on the receptor signaling in the urothelium and the function of adrenergic b-receptors are demonstrated.

4.1.3.6 Work package 7: Correlation between urodynamics and genes

A first objective, to establish standard protocols for acquisition of human bladder tissue, with defined inclusion criteria for patients and routines for sample handling was critical for the progress of this work package aiming at correlating gene/protein expression with clinical data. A protocol for acquisition and inclusion criteria was developed together with a specific ethical application (**D7.1**). INComb also ensured that each group had access to human material for addressing the research objectives. All groups have secured approved ethical permits for the samples and procedures for handling human material (**D7:2**). The procedures for ethical applications have been very time consuming and complicated, and it has also been difficult to exchange human material between countries due to local regulations. This problem was solved since the participants in each country had contacts between preclinical-clinical groups and since efforts were made to coordinate and standardize the documentation of human samples.

A biobank was established with blood samples with patient data and including cystometry for genetic analyses (**D7:4; D7:5; D7:6**). However, the clinical situation in the engaged department was reorganized which delayed the deliverables. However, INComb had aceess to a second unique biobank (from materiel obtained in Nijmegen, Netherlands material) which was analyzed as describe below. This biobank material is now made available for participants and external collaborators vi INComb. This DNA bank contained >1000 men with lower urinary tract function from Radboud University (Nijmegen, Netherlands). We also studies a second DNA bank with 392 subjects population-based from the Herne (Germany) area, and included men with and without lower urinary tract symptoms. The analysis focusing on polymorphisms of the β_3-adrenoceptor and M_2 muscarinic receptor genes (**D7:4; D7:6)**.It was initially screened for novel mutations by sequencing of the entire coding region and some adjacent stretches of the gene in about 100 subjects representing the high and low end of the bladder compliance spectrum. This yielded the known Trp64Arg polymorphism, a hitherto unknown polymorphism of the coding region Leu306Phe, and four non-coding polymorphisms; the latter were in complete linkage with the TrpArg64 polymorphism. Based upon genotyping of the entire 1015 DNA samples no statistically significant associations could be detected with any of a list of >30 parameters of lower urinary tract function except for a weak association with prostate size, which no longer was significant after multiple-comparison correction. In a parallel effort all of these mutants plus a previously reported one, not detected in our DNA bank, were created and stably transfected them into HEK cells for radioligand binding and cAMP accumulation studies. Five chemically distinct agonists including two in clinical development had very similar effects on all mutated, including two double-mutant receptors. The same DNA bank was also screened for associations of lower urinary tract function with polymorphisms of the M_2 muscarinic receptor (a CA-repeat polymorphism in the promotor region, which had previously been shown to be associated with a doubling of receptor expression in airway cells). Six different alleles of this polymorphic site were found accounting for 13 distinct frequent genotypes. However, none of them was significantly associated with urinary tract function. To better understand the discrepancy between the reported airway and the urinary tract findings, a follow-up study was initiated in which prostate samples were collected from about 100 consecutive patients undergoing surgery for benign prostate hyperplasia. These men were genotyped for the CA-repeat polymorphism and compared presence of the polymorphism to the abundance of M_2 receptor mRNA. In addition mRNA levels for all 5 muscarinic receptor subtypes were determined to explore correlations of expression with age, prostate size and other parameters. In a second DNA bank obtained from a representative sample of community-dwelling men

associations with two polymorphisms in the CYP 19A1 and one in the CYP 3A4 gene were analyzed in relationship to International Prostate Symptom Score, prostate volume and prostate-specific antigen levels. While the polymorphisms in CYP 19A1 did not exhibit an association, men carrying one mutated allele of the CYP 3A4 gene had a slightly but significantly smaller prostate and lower prostate-specific antigen level.

A collection of human samples from urinary bladder from human subjects with defined normal and pathological cystometry profiles was obtained to correlate gene expression profiles of specific human bladder tissues (urothelium, submucosa, detrusor) with urodynamic and clinical parameters. (**D7:3; D7:7**). Two innovative approaches were implemented (1) use of laser capture micro dissection (LCM) to procure pure cell populations and (2) application of exon arrays to get high resolution whole genome expression profiles as well as information about possible changes in alternative splicing of genes. Using these platforms the transcriptome changes in the detrusor of clinically characterized patients with bladder outlet obstruction and non-obstructed controls were examined at the exon level. Cluster analyses of microarray (exon array) data revealed that patients or controls can neither be grouped according to symptoms (assessed with IPSS), nor to urodynamic criteria (assessed with Abrams-Griffiths nomogram, Schäfer nomogram and ICS nomogram). Generally, patients and controls did not cluster in distinct groups. A series of additional control experiments excluded methodological errors with the highly complex techniques applied in the set-up. The data thus imply that – at least in the patient cohort investigated – obstructed and non-obstructed individuals do not differ with regard to their detrusor expression profiles.

In summary, this work package has reached its objectives. The sample size (in the tissue biobank) is still somewhat small but an initial report is available and data collection is ongoing which indicates that further achievements and reports are expected also after the formal conclusion of the INComb project. Interestingly, in the initial analysis, no major differences were detected in the expression profiles between urinary bladder samples from patients with normal bladders and from patients with bladders with outflow obstruction. The analysis of two existing biobanks available within INComb have examined nucleotide polymorophisms the β_3-adrenoceptor and M_2 muscarinic receptor genes and identified a novel polymorphism

4.1.3.7 Publications from INComb

The respective deliverables are indicated after each publication

Original papers published or in press

Anderson UA, Carson C, McCloskey KD. 2009. KCNQ currents and their contribution to resting membrane potential and the excitability of interstitial cells of Cajal from the guinea pig bladder. *J Urol.* 182:330-6. (**D4:6**)

Antunes-Lopes T, Carvalho-Barros S, Cruz CD, Cruz F, Martins-Silva C. 2011a. Biomarkers in overactive bladder: a new objective and noninvasive tool? *Adv Urol* 2011:382431. (**D3:3**)

Arrighi N, Bodei S, Lucente A, Michel MC, Zani D, Simeone, Cunico SC, Spano PF, Sigala S 2011. Muscarinic receptors stimulate cell proliferation in the human urothelium-derived cell line UROtsa. *Pharmacol. Res*. 64: 420-425. (**D6:1**)

Barendrecht MM, Frazier EP, Vrydag W, Alewijnse AE, Peters SLM, Michel MC. 2009 The effect of bladder outlet obstruction on α_1- and β-adrenoceptor expression and function. *Neurourol. Urodyn*. 28: 349-355. (**D6:1, 6:3**)

Berges R, Gsur A, Feik E, Höfner K, Senge T, Pientka L, Baierl A, Michel MC, Ponholzer A, Madersbacher S. 2011 Association of polymorphisms in CYP19A1 and CYP3A4 genes with lower urinary tract symptoms, prostate volume, uroflow and PSA in a population-based sample. *World J. Urol*. 29: 143-148. (**D7:6**)

Boberg L, Poljakovic M, Rahman A, Eccles R, Arner A. 2011 Role of Rho-kinase and protein kinase C during contraction of hypertrophic detrusor in mice with partial urinary bladder outlet obstruction. *BJU Int.* 109:132-40 (**D4:3**)

Cernecka H, Ochodnicky P, Lamers WH, Michel MC 2012. Specificity evaluation of antibodies against human β_3-adrenoceptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* (in press) (**D6:1**)

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Charrua A, Avelino A, Silva A, Cruz F. 2010. TRPV1 in GU Disorders. *The Open Drug Discovery J*. 2:49-53. (**D3:1, M3:1, D3:5**)

Charrua A, Avelino A, Cruz F 2011b. TRPV1 e Dor Visceral. *Dor* 19:5-10. **(D3:5**)

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Coelho A, Dinis P, Pinto R, Gorgal T, Silva C, Silva A, Silva J, Cruz CD, Cruz F, Avelino A. 2010. Distribution of the high-affinity binding site and intracellular target of botulinum toxin type A in the human bladder. *Eur Urol* 57:884-90. (**D3:6**)

Cruz CD, Cruz F. 2011. Spinal cord injury and bladder dysfunction: new ideas about an old problem. *ScientificWorldJournal* 11:214-234. (**D3:3**)

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4.1.4 Potential impact

A primary impact of INComb is that the project has advanced research and increased knowledge in the area of lower urinary tract function and mechanisms of incontinence. The project has generated a significant amount of scientific information as evident from the extensive list of original publications (60 full published scientific papers). As pointed out, the current development of treatment of OAB is slow partly due the lack of information on the complex function of the urinary bladder. The project has characterized several components and processes in the bladder wall which can influence the contractile activity, e.g. the communication between urothelium-nerves-muscle, a link between pain/inflammatory signalling and the action of interstitial cells, effects of physical factors and several novel potential targets (cell signalling, interstitial cell receptors). Although the project has not identified a specific target or treatment to bring into commercialization or exploitation, several novel potential targets are identified, e.g. receptors on interstitial cells or in intercellular signalling. Several novel experimental techniques for measuring signalling compounds have been developed. We expect that further work based on this information can generate novel treatment possibilities. Some of the mechanisms involved in current therapies (botulinum toxin, β-agonists, muscarininc antagonists, TRPV acting compounds) have been examined and provide a basis for further understanding and improvement of the current treatments.

The project results have been disseminated in the scientific community at several scientific meetings as shown by the large number of meeting abstracts (80 abstracts from scientific meetings). The INComb consortium has also organized a successful scientific meeting in Stockholm 2011 in collaboration with another EU projects (TRUST "Training Young Urologists"), and with the Swedish Enuresis Academy (SEA), a Society promoting research and help to children with incontinence. The meeting also included interaction and support from the European Association of Urology (EAU) who also helped to disseminate the meeting information. Close interaction was initiated during the meeting with key companies in the therapeutic area (Pfizer, Astellas, AstraTec, Ferring). Some lectures were videotaped for educational purposes. The meeting was highly appreciated and a symposium issue in the Acta Physiologica including several high-level reviews is currently under publication. We expect that this issue will summarize current knowledge in the field.

INComb has contributed to training of both clinical and basic scientists in the field. The project has a strong translational focus and we hope that a long term result is that a new generation of urological scientists with a translational research focus (basic and clinical science) will contribute to this research area in the future. The training activities are evident from the participation of several post docs and PhD students in the project and from the elective study reports and PhD-theses resulting from INComb activities. We believe that the unique combination of basic and clinical research has stimulated the students. This was further supported by the activities at the International conference where INComb and the meeting organizers supported attendance of younger scientists. The INComb constellation with its complementary competences and competent research groups has also forged a very productive interaction that we believe will continue to contribute to the fields of basic and clinical urology in the future.

INComb scientists have published 39 scientific reviews including results from INComb activities. Some of these reviews focus on basic properties of the urinary bladder function, but a majority of these reviews are addressing key therapeutic issues of clinical relevance,

including overviews of current pharmacotherapy and clinical treatment of incontinence. In this context we would like to mention our colleague in INComb, Dr Clare Fowler, who has contributed with several reviews and participated in the project with excellent clinical studies. She was appointed Commander of the Brittish Empire (CBE) in 2012, in recognition of her work in neuro-urology.

INComb scientists have had extensive dissemination activities toward the general public and patient organisations. During our international meeting in Stockholm 2011, we sent out a press release and the meeting was visited by members of the press. We also invited the representatives from the neurologically disabled patient organisation in Sweden ("Neurologiskt Handikappades Riksförbund, NHR"), which has a specific section for persons with incontinence problems. We were very pleased that they attended the meeting and provided very valuable contribution to interactions and discussion. Mrs Järneberg contributed to our conference book by writing "From the incontinent patient´s perspective". She was subsequently interviewed by jounalists together with a highly renowned Swedish urologist Prof Magnus Fall. Prof Fall also gave a very appreciated open lecture at the conference on Urinary incontinence. Both Mrs Järneberg and Prof Falls abstracts were printed in Swedish and English in the program. Our colleague from Zürich (Prof Hubert John) has organized public evening lectures "Forum Urologi" during 2011 (5 seminars) and 2012 (4 seminars) directed towards patients and their partners/families and patient organisations (program below). Professor Clare Fowler is involved as a trustee of the charity TUF - The Urology Foundation http://www.theurologyfoundation.org/ and has contributed with an article on "Fowler's syndrome" to COB - Cystitis and overactive bladder foundation http://www.cobfoundation.org/page/fowlers-syndrome. Prof F Cruz and colleagues from Porto have had several activities toward the general audience and patient organizations. The participants from the Porto group have participated in more than 25 different dissemination activities including television programs, newspaper articles and radio programs.

4.1.5 Web page

The website is www.INComb.eu

The INComb project as well as the list of beneficieries and their contact information is presented on the homepage.

4.2 Use and dissemination of foreground

The dissemination measures, scientific papers and foreground are uploaded at the Comission homepage and are presented in the Tables of Section A and B.