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REGENERATIVE MEDICINE AND REGISTER STUDIES RELATED TO BLADDER EXSTROPHY

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Regenerative medicine and register studies related to bladder exstrophy

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ABSTRACT

Bladder exstrophy is a rare congenital malformation affecting both boys and girls. The malformation involves the bladder, the urethra, genitalia, and pelvis. The child presents with a defect of the lower abdominal wall exposing an open bladder.

In this thesis, the field of regenerative medicine is explored with the aim of improving the surgical treatment of malformations where there is a lack of tissue, such as bladder exstrophy when a bladder conduit is sometimes needed for bladder catheterization. The thesis also includes register studies for further understanding of the prevalence, morbidities, the quality of life, and risk factors associated with bladder exstrophy.

Studies I and II were conducted in a porcine animal model with the aim of constructing a functional autologous bladder conduit to be used for catheterization. Both studies were aimed at using regenerative medicine techniques with minimal *ex vivo* handling. Bladder tissue was obtained and minced for autologous tissue transplantation in order to analyze the regenerative capacity of the mucosa, the urothelium, and the muscle, the detrusor. Animals were housed up to five weeks post transplantation. In Study I, functional bladder emptying tests were performed by catheterization through the neo-regenerated conduit and CT scans were used for anatomical evaluation. In Study II, detrusor muscle expansion was studied *in vitro* and *in vivo* for evaluation of the regenerative capacity after mincing. In conclusion, bladder wall mincing techniques could be a valuable tool for autologous tissue expansion, which can be prepared at the operating table as a one-step surgical procedure.

Study III was conducted as a matched case-control study using Swedish national population-based registers. One-hundred-and-twenty cases of bladder exstrophy were identified between 1973 and 2011. The prevalence was 3:100 000, which was stable over time. In contrast to other publications, an equal male-to-female ratio was found. Birth descriptive data were comparable to those of controls, and bladder exstrophy occurred most often without any other major associated malformations. Congenital inguinal hernia and undescended testis were, however, more prevalent than in the general population. The only potentially associated maternal risk factor was advanced maternal age.

Study IV was conducted as a matched cohort study between 1952 and 2011. We used Swedish national population–based registers to assess morbidity, mortality, and such social

parameters as partnership, fertility, and education. One-hundred-and-eighty cases were identified for analyses of morbidity and mortality and 124 of these cases were individuals over 18 years of age and thus were available for further social analyses. The incidences of bladder cancer and psychiatric disorders were comparable to those of the general Swedish population. Mortality was low. Sensory neurogenic hearing disorders indicated a possible genetic association in a few cases. No differences concerning partnership could be established, but significantly fewer cases conceived biological children. The educational level was high overall, with male cases reaching a slightly lower level, and females a higher level of the highest level of education compared to controls.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Medfödda missbildningar kräver ofta kirurgi tidigt i livet. Det är vanligt att behandlingen försvåras av att det saknas vävnad. Vanligtvis används omkringliggande vävnad eller transplantat från mer avlägset belägen vävnad. Dessa behandlingar är ofta förenade med komplikationer då den vävnad som används för rekonstruktion inte är skapad för samma funktioner som den vävnad som ersätts.

Regenerativ medicin innebär att man skapar nya vävnader inuti kroppen med hjälp av kroppsegna celler och bärarmaterial som transplanteras till mottagaren. Fördelen med metoden är att man kan designa transplantaten efter patientens behov och att transplantaten inte avstöts tack vare att cellerna är kroppsegna. En nackdel är att metoden fortfarande är relativt dyr och kräver högspecialiserad personal och laboratorium. Fortsatta studier krävs även för att ta fram ett bärarmaterial med optimala funktionella och fysikaliska egenskaper för transport av näringsämnen samt inväxt av nerver och blodkärl.

Inom barnurologin förekommer ofta medfödda missbildningar med avsaknad av vävnad såsom exempelvis vid blåsexstrofi. Utveckling inom regenerativ medicin avseende rekonstruktiv kirurgi för blåsförstoring eller för att skapa kroppsegna vävnadsrör för kateterisering av urinblåsan vore ett tilltalande alternativ.

Blåsexstrofi är en ovanlig medfödd slutningsdefekt av nedre delen av bukväggen, urinblåsan och urinröret, könsorgan och bäckenet som leder till att urinblåsan ligger öppen nedanför naveln. Pojkar föds med epispadi, där urinröret ligger öppet på en kluven penis, och flickor med delad klitoris och en kortare vagina. Blåsexstrofi räknas ofta till ett spektrum av medfödda missbildningar; epispadi, blåsexstrofi och kloakexstrofi, där blåsexstrofi är den vanligaste. Förekomsten är cirka 3:100 000 och uppstår både hos flickor och pojkar. Den underliggande orsaken är multifaktoriell med både genetiska och miljöpåverkande faktorer. Defekten kräver upprepad kirurgi från nyföddhetsperioden till vuxen ålder.

Den här avhandlingen syftar till att undersöka om det går att skapa en kateteriserbar kanal av finfördelad blåsvävnad från urinblåsan till hudytan, utan att behöva odla celler i laboratoriemiljö. Studierna har gjorts i en djurmodell på gris men även som förberedande studier i cellodlingslaboratorium. Avhandlingen syftar även till att öka kunskapen om blåsexstrofi gällande förekomst, sjukdomspanorama och eventuell påverkan på sociala parametrar senare i livet. Nationella svenska register har legat till grund för dessa studier.

Studie I och II utfördes på gris med förberedande studier i laboratoriemiljö i studie II. Finfördelad vävnad från urinblåsa transplanterades till ett syntetiskt rör som sedan inopererades i gris. Syftet var sedan att ta bort röret efter att nybildning av vävnad skett. I studie I kunde vi genom denna metod skapa en tunnel mellan urinblåsa och hud som kunde användas för kateterisering av urinblåsan för tömning av urin. Tunneln skapades av finfördelad slemhinna från urinblåsan. Cellerna i den finfördelade vävnaden kunde tillväxa och migrera och skapade därmed en sammanhängande vävnad. Cellerna satt dock relativt löst fast vid den underliggande vävnaden och lossnade lätt vid kateterisering. I studie II var därför hypotesen att tunneln kunde stärkas med hjälp av ett muskellager från urinblåsan. Studier i laboratoriemiljö visade att finfördelad muskelvävnad från urinblåsan växte bra. I grismodellen växte celler från finfördelad muskel likvärdigt med finfördelad slemhinna från urinblåsa men vid samtidig transplantation av muskel och slemhinna utvecklades inte de transplanterade cellerna från slemhinnan. Vi kunde därför inte bekräfta hypotesen om att en tunnel kunde stärkas genom att samtidigt transplantera både slemhinna och muskel, å andra sidan kunde vi visa att finfördelad blåsmuskel kunde expandera i kroppen utan att behöva odlas i laboratoriemiljö.

I studie III utfördes en nationell registerstudie på blåsexstrofipatienter födda i Sverige mellan 1973-2011. Alla identifierade fall matchades med fem kontroller avseende kön och födelseår. Vi använde oss av nationella register och syftet var att beskriva förekomsten och födelseegenskaper inklusive andra medfödda missbildningar hos barn födda med blåsexstrofi samt eventuella riskfaktorer hos mödrarna. Etthundratjugo patienter med blåsexstrofi identifierades. Förekomsten var 3:100 000 levande födda barn och det var lika vanligt hos flickor som hos pojkar. Missbildningen brukar annars beskrivas vanligare hos pojkar. Förekomsten var även likvärdig under tidsperioden. Blåsexstrofi var oftast inte förenat med andra större missbildningar men medfött ljumskbråck och icke nedstigna testiklar var vanligare jämfört med de matchade kontrollerna. Den enda potentiellt associerade riskfaktorn hos mödrar var hög ålder.

Studie IV var en uppföljningsstudie på blåsexstrofipatienter. Nationella register användes för att identifiera fall mellan 1952-2011. Fallen matchades med fem kontroller avseende kön och födelseår. Vi identifierade totalt 180 fall där 124 fall var över 18 års ålder. Syftet var att utvärdera dödlighet, förekomst av cancer, psykiatriska diagnoser och hörselnedsättning samt att utvärdera förekomst av partnerskap, biologiska barn och utbildning. Varken cancer i urinblåsan eller psykiatriska diagnoser var vanligare än hos den svenska befolkningen. Dödligheten var låg. Medfödd hörselnedsättning förekom hos några få blåsexstrofipatienter

vilket indikerade en möjlig genetisk orsak till blåsexstrofi. Det förelåg inga skillnader vad gäller partnerskap men det förelåg en skillnad i antalet biologiska barn, speciellt hos kvinnorna. Utbildningsnivån var generellt hög där män nådde en något lägre andel och kvinnorna en något högre andel när vi tittade på högsta utbildningsnivån.

PREFACE

When doing my clinical rotation in adult urology, I once had a young man at my outpatient clinic. He was self-confident, vital and looking forward to his future life as most young men of his age. He was born with bladder exstrophy and with surgery he had been continent since childhood. He was enjoying his life at full. He loved skiing and two months earlier he had been on a skiing trip with friends in Northern Sweden. Unfortunately, he was in a very bad accident. He crashed on his skis going downhill and lost consciousness. Since he was closer to Norway, a helicopter flew him to Trondheim. An x-ray of his pelvis was administered in the helicopter, and the personnel interpreting the film clearly identified an open book fracture of the pelvis. Since there was no appearance of blood at the meatus, a large caliber catheter was introduced but could not be passed. Later at the hospital he received a supra pubic catheter. His recovery was uneventful but when retrieving his medical charts from Sweden they soon learned that there was no open book fracture of the pelvis but a normal pelvis of someone born with bladder exstrophy. The attempt to introduce the large caliber catheter had severely damaged his neourethra and he was now forced to use a supra pubic catheter.

This young man was probably one of the most challenging patients in the outpatient clinic, and I therefore consulted my senior colleges. They were puzzled. The cystoscopes at the clinic were too large and who was the best clinician to perform the procedure. No one stepped forward. He was later referred both to pediatric urology, to have the cystoscopy performed with the correct instruments, and to an adult urologist, with more interest in this rare patient group. Unfortunately, I do not know the outcome of this young man but I can only hope that his life is back to where it was before the accident, in every perspective.

This young man has been on my mind since I met him, and I hope that my work and this thesis will share a light on all the patients born with bladder exstrophy and possible future surgical interventions to improve their quality of life.

LIST OF SCIENTIFIC PAPERS

I. **Reinfeldt Engberg G**, Lundberg J, Chamorro CI, Nordenskjöld A, Fossum M.
Transplantation of autologous minced bladder mucosa for a one-step reconstruction of a tissue engineered bladder conduit.
Biomed Res Int. 2013;2013:212734.

II. **Reinfeldt Engberg G**, Chamorro CI, Nordenskjöld A, Fossum M.
Expansion of submucosal bladder wall tissue *in vitro* and *in vivo*.
Biomed Res Int. 2016;2016:5415012.

III. **Reinfeldt Engberg G**, Mantel Ä, Fossum M, Nordenskjöld A.
Maternal and fetal risk factors for bladder exstrophy: A nationwide Swedish case-control study.
J Pediatr Urol. 2016 Jul 15. pii: S1477-5131(16)30135-8.

IV. **Reinfeldt Engberg G**, Mantel Ä, Fossum M, Nordenskjöld A.
Long-term outcome of living with bladder exstrophy in Sweden: A nationwide matched cohort study.
Manuscript.

LIST OF ABBREVIATIONS

ART	Assisted reproductive technology
BE	Bladder exstrophy
BEEC	Bladder exstrophy epispadias complex
BNP	Bladder neck plasty
CE	Cloacal exstrophy
CI	Confidence interval
CIC	Clean intermittent catheterization
Ck	Cytokeratin
CPRE	Complete primary repair of exstrophy
CT	Computed tomography
GAG	Glykosaminoglycan
Fr	French, 1 Fr = 1/3 mm
H&E	Hematoxylin and eosin
ICD	International Classification of Diseases
ICS	The International Continence Society
IVF	*In vitro* fertilization
MBR	Medical Birth Register (Medicinska födelseregistret)
MSRE	Modern stage repair
NPR	National Patient Register (Patientregistret)
OEIS	Omphalocele-exstrophy-imperforated anus-spina bifida

OR	Odds ratio
PCL	Polycaprolactone
PIN	Personal identification number (Personnummer)
SCB	Statistics Sweden (Statistiska centralbyrån)
SD	Standard deviation
Shh	Sonic hedgehog
SNBHW	the Swedish National Board of Health and Welfare (Socialstyrelsen)
UP	Uroplakin

CONTENTS

1 INTRODUCTION

Having a child with a rare congenital malformation is, by any measuring stick, overwhelming. The parents probably have more questions than we, as clinicians, can ever imagine. What we do and say may affect the family and the child for their entire life. Whatever we do must last for 80 years. We have to manage parents and their expectations in dealing with fetal outcomes, possible interventions, postnatal outcomes, possible surgical interventions and long-term outcomes, both surgical and psychosocial. Is there a genetic background or are there maternal risk factors affecting the outcome? Are there other associated anomalies? Will it happen to a second child? Will there be lifelong problems? What kind of surgeries or other optional treatments are available? How will surgery affect my child? What about social relations? Are there solutions to the problems?

Whenever there is a problem, we look for a solution. For a clinician, the ultimate goal is to be in agreement with the patient and to make use of long lasting solutions to their problems. This thesis is aimed at elucidating bladder exstrophy, a rare congenital malformation affecting the urinary bladder, the urethra, genitalia, and pelvis due to a defect in the lower abdominal wall involving an exposed open bladder, and the possibility of the application of regenerative medicine, the technique of rebuilding tissues and organs, for reconstructive bladder surgery. It is only by bringing science and clinic together that we can succeed in both fields. It all starts with understanding normal and abnormal development as well as structures and signals within the body.

1.1 EMBRYOLOGY

1.1.1 Development of the urinary bladder and the urethra

The urogenital system can be divided into two separate functional systems, the urinary system and the genital system. However, in the embryo and during organogenesis, the two systems are closely intertwined. Both develop from a common ridge of intermediate mesoderm, the urogenital ridge, along the posterior wall of the abdominal cavity, which develops into three tubular nephric structures (from head to tail) – the pronephros, the mesonephros, and the metanephros.

The mesonephros and the mesonephric ducts are derived from the intermediate mesoderm early in the 4th gestational week when the pronephric system regresses. The mesonephros is a transitory structure in which most of the mesonephric structures regress, but a pair of

mesonephric ducts, the Wolffian ducts, persists and opens into the cloaca. By the 5th week, a pair of ureteric buds sprouts from each of the distal mesonephric ducts, marking the beginning of the development of the ureters. The ureteric buds also induce the overlying sacral intermediate mesoderm to develop into the definitive kidneys. The future gonads develop in close proximity to the developing kidneys from the mesothelium of the posterior abdominal wall, the underlying intermediate mesoderm called the gonadal ridge and the primordial germ cells.

During gestational weeks 4-7, the cloaca, the distal extension of the hindgut, is divided by the urorectal septum. This marks the development into the ventral urogenital sinus and the dorsal part rectum and the anal canal. The superior ventral and largest part of the urogenital sinus, the vesical part, forms the urinary bladder, at first continuous with the allantois, a diverticulum of the hindgut extending into the umbilicus. When the allantois is obliterated only a thick fibrous cord is left, the urachus, connecting the apex of the bladder with the umbilicus. This later transforms into the median umbilical ligament in the adult. In females, most of the urethra is also derived from this part. The inferior ventral part of the urogenital sinus gives rise to the pelvic part which, in males, forms the prostatic and membranous parts of the urethra and, in females, the membranous part of the urethra. The most inferior region is located in the phallus, which develops differently in males and females. It forms the penile urethra in males and the vestibule of the vagina in females (Figure 1).

During differentiation of the cloaca, the caudal parts of the mesonephric ducts are united with the wall of the urinary bladder, thus causing the ureters to enter the bladder separately. The root of the mesonephric duct opens into the posterior wall of the developing bladder and fuses into the bladder to form the trigone of the bladder. Since both the mesonephric ducts and the ureters are of mesodermal origin, the trigone also becomes of mesodermal origin. The mucosa of the trigone is later replaced by overgrowth of endodermal epithelium, thereby causing the whole bladder to be lined with the same epithelium. Later, in the 12th week, splanchnic mesoderm associated with the hindgut forms the smooth muscle of the bladder wall.

When the kidneys ascend later in the development, the orifices of the ureters move cranially and the mesonephric ducts move close together to enter the prostatic urethra in the male forming the ejaculatory ducts and the vas deferens. In females, this part will degenerate. Instead, the female genital ducts arise from the paramesonephric ducts, also called Müllerian ducts, which can be understood to be a derivative of the pronephros and are therefore

separated from the process of mesonephric development. The fused paramesonephric ducts give rise to the fallopian tubes, the uterus and the upper part of the vagina.[1-6]

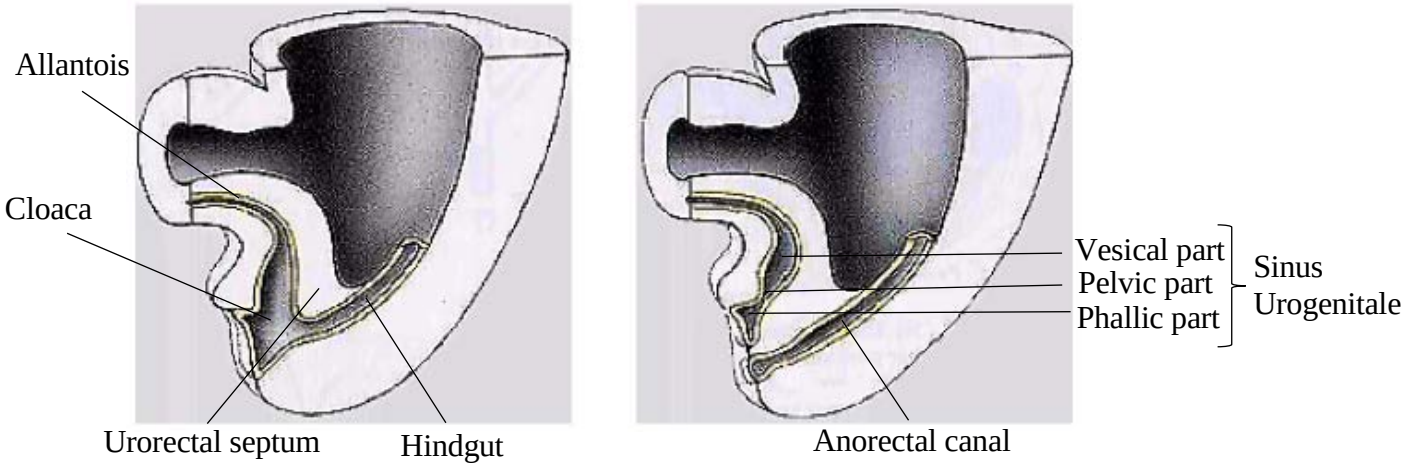

Figure 1. Development of the cloaca. Pictures from www.slidshare.net, modified by the author.

1.2 ANATOMY AND HISTOLOGY

1.2.1 Urinary bladder

The basic function of the bladder is to store urine until voiding. It is anatomically constructed to refrain the urine from backing up the ureters. The ureters enter the wall of the bladder obliquely, and by these means, an increase of bladder pressure, as in voiding, allows the bladder mucosa to close the ureteral orifices so as to pass urine only through the urethra.

Voiding is controlled by an involuntary sphincter located adjacent to the base of the bladder. The sphincter opens when the bladder is full due to the action of autonomic innervation and parasympathetic stimuli. In addition to this, there is a voluntary sphincter, the external urethral sphincter, located in the perineum of the pelvic floor. This sphincter allows the voluntary retention of the urine in the bladder until voiding is acceptable. In adult females, the urethra is short, 4-5 cm long, and is an independent system while, in males, the urethra is longer, 15-20 cm in length, and is shared with the reproductive system.

Microanatomically, the bladder is constructed from a transitional epithelium, i.e. urothelium, and a muscular wall, the detrusor. The highly specialized urothelium lines the entire urinary tract from the renal pelvis to the urethra.

The walls in the urinary system are formed by three different layers:

a) The mucosa: the inner layer consists of the urothelium with a supporting connective tissue, the *lamina propria*, where blood vessels and nerves are found. The *lamina propria* can be subdivided into two layers: a superficial, dense, irregular collagenous connective tissue and a deeper, looser layer composed of a mixture of collagen and elastic fibers. Between the layer of the mucosa and the *lamina propria* is the basal membrane, a base for the urothelium.

b) The muscularis: the muscular layer, the detrusor, is composed of three different interlaced layers of smooth muscle: inner longitudinal, middle circular, and outer longitudinal. The middle circular layer extends below the bladder to form the internal sphincter around the urethra. Contraction of the detrusor muscle is made possible by stimulation of the muscle cells by the parasympathic nervous system, which can be initiated by voluntary control after infancy.

c) The connective tissue: a thin connective tissue covering, the *adventitia*, houses blood vessels and nerves supplying the detrusor. It also holds each ureter towards the posterior abdominal wall, separating it from the overlying peritoneum. Certain regions of the adventitia are covered with a serosa layer as part of the peritoneum[7-9] (Figure 2).

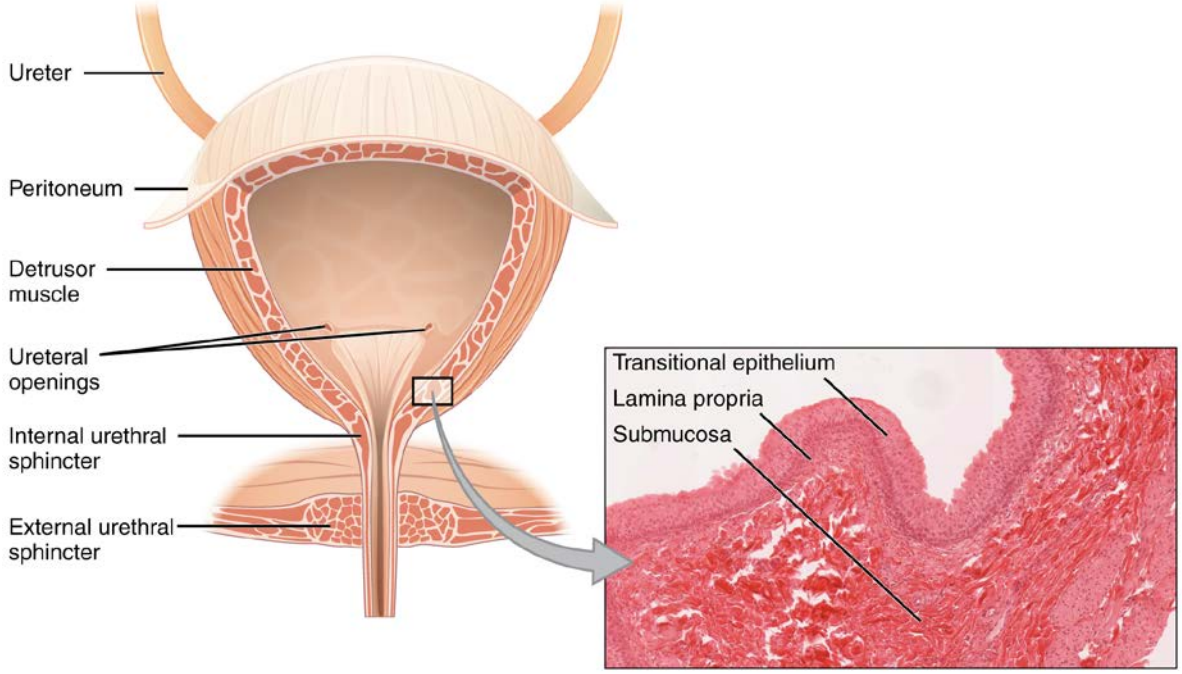

Figure 2. Left: Anatomy of the female bladder. Right: Histological cross-section demonstrating mucosa: transitional epithelium and lamina propria, and submucosal structures: muscularis and connective tissue. From Wikipedia, the free encyclopedia, Urinary bladder. http://creativecommons.org/licenses/by-sa/3.0/

1.2.2 Urothelium

The urothelium consists of different cell layers comprised of cells with many different functions. In an empty bladder, the mucosa is folded forming rugae, or folds, consisting of many layers of cells, but as the bladder is filled, the epithelial cells are flattened and the number of cell layers decreases. The mucosa of the trigone, where the ureters enter and the urethra forms, is always smooth and without folds. The epithelium lining the innermost part of the bladder consists of umbrella cells. In an empty bladder, these cells are large, round and dome-shaped and occasionally binucleated. These cells are impermeable and serve as an osmotic barrier ensuring that hypertonic urine does not come into contact with surrounding isotonic tissue and blood. In addition, toxic molecules remain in the urine and are not taken up by the body again[7-9] (Figure 3).

There is a thick layer of glycoproteins and proteoglycans, glycosaminoglycans (GAGs), on the surface of the urothelial umbrella cells. They constitute a mucosal coating that acts as a barrier against solutes found in the urine, thereby protecting the cells underneath from harmful substances. Injury to GAGs may be the first step in the genesis of bladder disease.[10]

The umbrella cells are covered by highly specialized scallop-shaped membrane plaques consisting of membrane proteins, uroplakins and integral membrane proteins. The four different identified uroplakins (UPIa, UPIb, UPII, and UPIIIa) are widely regarded as urothelium-specific markers of terminal urothelial cytodifferentiation and are expressed only in terminally differentiated urothelial cells. Uroplakins mediate signal transduction in the regulation of cell development, activation, growth, and motility. They probably play a role in normal bladder epithelial physiology, in regulating membrane permeability of the superficial umbrella cells, or in stabilizing the apical membrane through cytoskeletal interactions. The apical membrane is believed to be involved in strengthening the umbrella cells, and also in enhancing the inner bladder membrane's ability to stretch, thus preventing these cells from rupturing during bladder distension.[11-13]

The several cell layers in the middle are composed of polyhedral cells, cells with multi-sides, straight edges, and sharp corners. There are tight junctions, or *zonae occludentes*, between these cells, joining adjacent cells together to form an impermeable barrier as well as being important in intracellular signaling.

The epithelial cells lying on the basal membrane are small, either low columnar or cuboid cells, but when migrating to a more superficial position they become larger and more pear-shaped.

The cells within the urothelium have the ability to change their size and form so as to extend when the bladder is stretched, and to fill out when the bladder is relaxed. Through this mode of action, the cell layers of the urothelium may be approximately four to eight cell layers thick with different physiological adaptions (Figure 4).

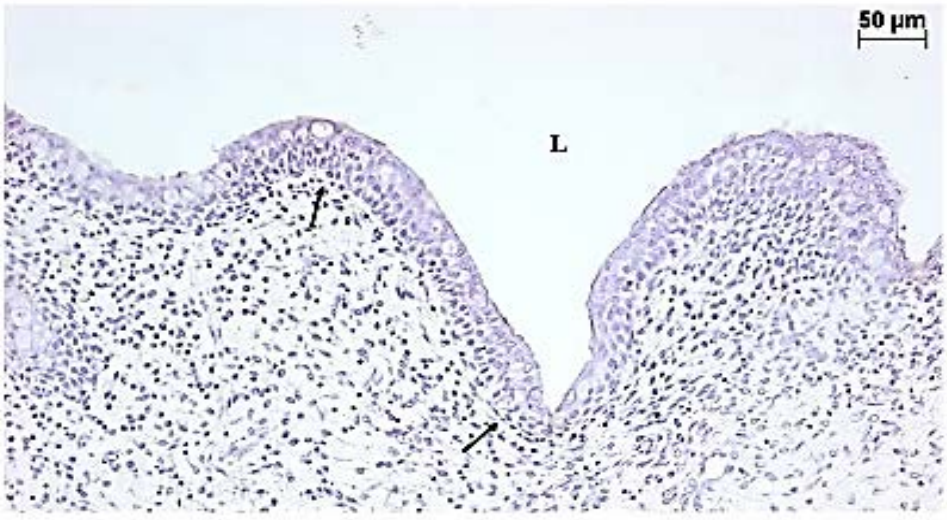

Figure 3. Photomicrograph of porcine urothelium. The arrows point at the basal membrane with cuboid and columnar cells on the basal membrane. Large multinucleated cells closest to the bladder lumen (L), (CkMNF116, x100).

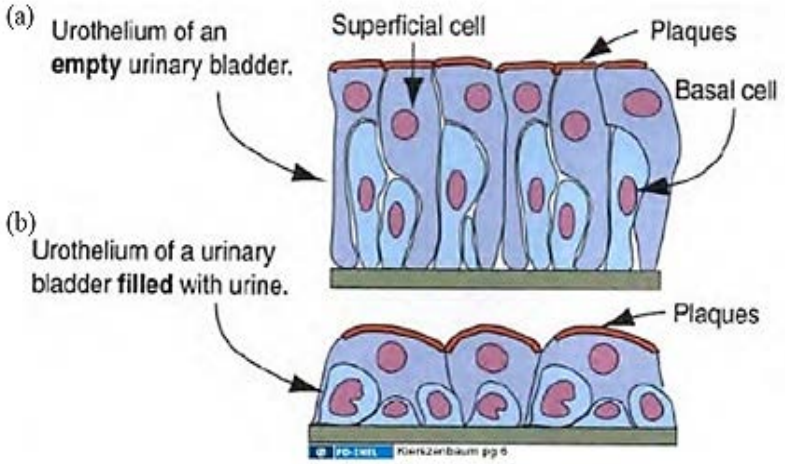

Figure 4. Urothelium in (a) a relaxed bladder and (b) a stretched bladder.
http://www.slideshare.net/openmichigan/090808-epithelialtissue.
http://creativecommons.org/licenses/by-sa/3.0/

1.3 REGENERATIVE MEDICINE

Tissue engineering, as a field of medicine, was defined in 1987, with reference to the practice of combining scaffolds, cells, and biologically active molecules into functional tissues.[14]

The term regenerative medicine includes the field of tissue engineering and also incorporates research using the body as a bioreactor to rebuild tissues and organs.

In 1958, Meek introduced a theory concerning the regenerative capacity of skin epithelium by showing that epithelial cells could regenerate from the edge of a cut surface by migration and cell division, leading to the idea that by dividing a square of skin epithelium into two equal sizes, the regenerative capacity could be increased[15] (Figure 5).

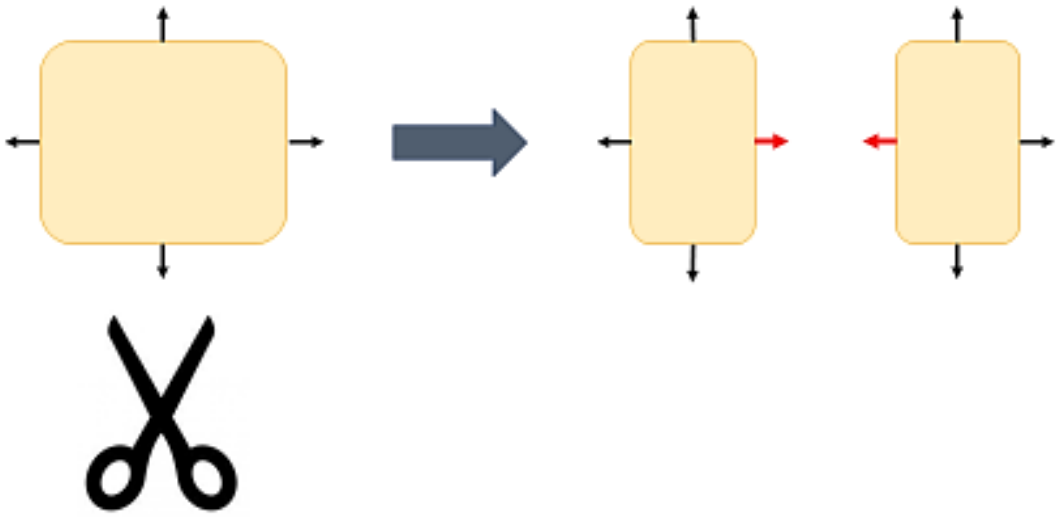

Figure 5. Drawing illustrating the theory of Meek. Red arrows indicate areas of increased regenerative capacity after division.

Applications of tissue engineering and regenerative medicine in humans are historically mostly used clinically in burn injuries with cultured epidermal autografts to cover severely damaged skin.[16-18] Autologous transplanted cells have also been used in clinical bone regeneration to repair cartilage.[19-21]

Urothelium has also been shown to regenerate from the wound edges in a way similar to that of epithelium.[22-23] However, regeneration of the bladder wall tissue beneath the urothelium has been less well described. *In vitro* expansion of smooth muscle cells is made possible by

using explant techniques[24], suggesting that migration to the edge of a wound before proliferation is a possible mode of action, and that the development might be through cell signaling. In line with this, smooth muscle cells seeded on urethral scaffolds have demonstrated the possible strengthening capacity of the urothelial structure over time.[24-26]

In 1999, the first successful attempt to create a tissue engineered neourinary bladder was reported. The bladder was created from canine native bladder biopsy specimens with *in vitro* culturing of urothelial and smooth muscle cells and then seeded onto a preformed bladder-shaped polymer and further cultured *in vitro*. Native canine bladders where then replaced with the tissue engineered neobladder *in vivo* with a follow up of one year with good functional results.[27] Later, the same method of autologous bladder transplantations was applied in humans using a biodegradable mould. In 2006, the follow-up results after five years were reported and demonstrated that the method was feasible.[28]

1.3.1 Signaling systems of the urinary bladder

The signaling systems between cells in the different layers of the urinary bladder wall are not yet fully understood, but it has been shown that co-culturing human smooth muscle cells and urothelial cells *in vitro* on synthetic hydrogel matrices, as well as on intestinal submucosa, creates a positive environment for proliferation of both cell types.[25,29-30]

The ability of the urinary bladder to maintain an intact barrier, despite volume and pressure alterations, is largely dependent on features of the surface layer of the urothelium with its transitional epithelium including umbrella cells as the top layer cells facing the lumen.

There is increasing evidence that all the layers of the urinary bladder urothelium exhibit specialized sensory properties that play a key role in the detection and transmission of both physiological and nociceptive stimuli, including the ability to respond to chemical, mechanical and thermal impulses that probably communicate the state of the urothelial environment to the underlying nervous and muscular systems.[31] The signaling systems within the urothelium in the bladder is also considered to be of vital importance for smooth muscle differentiation.[32-33]

1.3.2 Urothelial cell culturing and harvest techniques

Urothelial cells may be cultured using specific urothelial cell culturing medium. Nowadays, this is readily commercially available. The cells are cultured in an incubator at 37°C for

human cells and preferably approximately 36°C for porcine cells since the pig has a slightly lower body temperature. The cell medium has to be changed every other day and the cells checked for viability and proliferation, with splitting of the cell culture when sub it is confluent, for further expansion. The splitting of the cell culture into preferably three new wells marks a new passage for the cells.

1.3.2.1 Single-cell suspension

Single-cell suspension may be obtained from biopsies or from bladder washings.[34-36] The tissue or urine sample must then undergo enzymatic procedures in an accredited laboratory to produce a single-cell suspension, and then further cultured *in vitro* for a number of weeks in order to produce the right amount of cells needed for transplantation. Cells must adhere to the plastic bottom in order not to adhere to each other forming non-replicating clusters of cells. This can be mediated, basically through feeder cells and adding different substrates to the cell culture media.[37] The method of culturing cells in single-cell suspensions is, however, rather time-consuming, expensive, and comes with many regulations if intended for transplantation back to the patient. In order to obtain large enough quantities for transplantation, the cells have to go through several passages, basically aging the individual cells. This has been shown to result in alterations in cell proliferative capacity, which may ultimately influence the viability of the transplant.[38] One also has to take account of the fact that when handling the cell cultures over time, they become exposed to external environmental factors, such as bacteria, viruses and perhaps other cytotoxic agents.

1.3.2.2 Explant technique

The explant technique is one of the oldest methods for obtaining cell cultures. With this technique, cells are cultured *in vitro* from tissue samples, such as biopsies, on plastic containers in medium. The cells will migrate from the tissue biopsy and then proliferate in the culture well.[39] This method is, like the previous one, time-consuming and subject to the same regulation as for pharmaceuticals, if intended for patient transplantation.

1.3.2.3 Minced tissue

By using minced tissue, a somewhat larger specimen is used to produce many small particles that can either be cultured in plastic wells or directly incorporated and cultured in a carrier material. The method is faster with respect to extra-corporal processing, and cell expansion occurs according to the theory of Meek, by cell migration and proliferation, and in analogy

with the explant culturing technique. The mincing technique allows an expansion of the cell proliferative capacity by creating a larger surface from where migration can take place. The method is less time-consuming, without any need for *ex vivo* cell expansion in specialized cell culture facilities and is therefore less expensive, and may also produce a larger transplant from the same amount of tissue specimen. If incorporated into a carrier material from start, the transplant can be directly available for retransplantation to the patient. The backside of this is that, for mincing, the tissue specimen must be larger which could pose a problem with a lack of tissue.[22,40-43]

1.3.3 Carrier materials and cell transplantation

Most cells need to be transported on carrier materials, scaffolds, to remain as continuous cell sheets and to allow surgical handling. The carrier material should constitute a bed for the supply of nutrients and comprise optimal physiological and functional capacities mimicking the transplantation environment, as well as promote adhesion at the donor site.

Scaffolds may be acellular by virtue of their own property, such as collagen matrixes and artificial materials, or they may be composed of tissues that have been decellularized, mostly non-urologic (skin, gastrointestinal segments or mucosa).[44] The aim of all scaffolds is to constitute a micro skeleton that guides cell migration and ingrowth of vessels and nerves in a three-dimensional manner. Because of the scaffold's acellular property, rejection is avoided.

As transplants, scaffolds can then either be biodegradable or non-biodegradable. Biodegradable scaffolds are degraded over time *in vivo*, leaving only the neo-regenerated tissue, while non-biodegradable scaffolds remain as a structural component of the transplant.

Various scaffolds have been used, but until now, no one has introduced a scaffold that includes all the necessary properties while being easy to handle, inexpensive, and clinically available for reconstruction of bladder tissue.[45-49]

The cells can then be cultured on the scaffold *in vitro* before use as a ready-to-work transplant for the recipient, as in burn injuries, or they can be transplanted with minor *ex vivo* processing as autologous transplants *in vivo*. By these means, the patient's own body functions as a natural bioreactor supplying the transplant with nutrients and physical properties in a natural 37°C environment, where take of cells, proliferation, and further tissue regeneration can occur.

1.3.4 Routine and immunohistochemistry staining as cell markers

In cell and tissue culturing, cell authentication must be ensured by morphological studies, as well as expression of cell protein markers, which can be stained. There are numerous commercial products available for routine histology and antibodies for immunohistological staining. Different staining or antibodies detect and enhance different structural morphologies, cells, and proliferation activity. Some of them are very specific, while others detect more than one type of cell. Below are a few of these and the ones that were most frequently used in in this thesis (Figure 6).

1.3.4.1 Routine histology

Hematoxylin and Eosin (H&E) is used as a routine staining to primarily clarify structural morphology within the material. The staining is a combination of a basic and an acid dye where the acidic eosin, stains basic structures red or pink, and the basic hematoxylin, stains acid structures blueish. Most of the proteins in the cytoplasm are basic and therefore cytoplasm will appear red or pink. Cell nuclei and ribosomes are acidic and will therefore stain blue.

Masson's Trichrome, also used as a routine staining, distinguish cells from surrounding connective tissue. Muscle fibers produce red keratin; collagen and bone blue or green; cytoplasm light red or pink and cell nuclei dark brown to black. This staining is valuable for identifying mature connective tissue from granulation tissue and newly regenerated collagen.

Van Gieson is sometimes used as a routine staining where nuclei stain blue or black, collagen red and other tissue components yellow.

1.3.4.2 Immunohistochemistry

CkMNF116 reacts with cytokeratins 5, 6, 8, 17 and probably also 19. Cytokeratins are found in the intracytoplasmic cytoskeleton of epithelial tissue and are of two types, I acid and II basic/neutral, which interact in pairs. The antibody shows an especially broad pattern of reactivity with human epithelial tissue from simple glandular to stratified squamous epithelium and can be used in the detection and classification of normal and neoplastic cells of epithelial origin.

Ck5, a type II cytokeratin consisting of basic/neutral proteins which are arranged in pairs of different types of keratin chains co-expressed during differentiation of simple and stratified epithelial tissues. Ck5 is specifically expressed in the basal layer of the epithelium.

UpIIIa is specific for urothelial cells. In normal urothelium, superficial umbrella cells express four major integral membrane proteins, uroplakins UPIa, UPIb, UPII, and UPIIIa, which compose urothelial plaques.

Alpha-actin is expressed in skeletal muscle and is one of six different actins that have been identified. Actins are involved in cell motility, structure, and integrity. Alpha-actin may also be detected in fibroblasts.

Ki-67 is strictly associated with cell proliferation and may therefore be seen in any proliferating cell. Ki-67 protein is present during all active phases of the cell cycle (G_1, S, G_2, and mitosis), but is absent in resting cells (G_0).

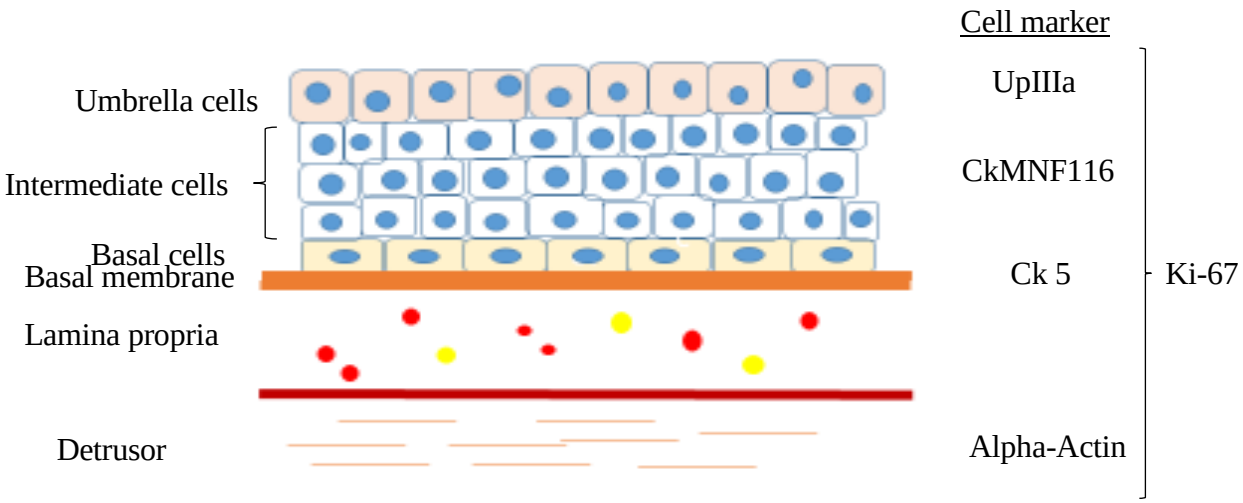

Figure 6. Drawing of the structures of the urinary bladder demonstrating where different cell markers may be detected.

1.4 BLADDER EXSTROPHY

1.4.1 History

The term bladder exstrophy (BE) originates from the Greek word *ekstriphein*, meaning “turn inside out” or *ectopos* “out of position”, and is also known as ectopia vesicae in older literature and diagnostic coding systems, which is the latin term for describing the malformation.[50] François Chaussier was the first to use the term “exstrophy” in the year 1780.[51]

The first known inscription of BE is noted on a clay tablet where human birth defects, including extrophy of the bladder, are recognized. The tablet was part of the great library of King Ashurbanipal, who reigned during the neo-Assyrian period (668-c.630 BC) in Northern Iraq at his capital, Nineveh. The library includes texts of scientific and administrative value, as well as royal inscriptions, now found at the British Museum, London, UK[52] (Figure 7).

Figure 7. K.3686, tablet, Library of Ashurbanipal, British Museum, Middle East Section.

It was not until 1597 that the second known description of BE was presented by Schenkii von Grafenberg, Frankkfurt, in a context of observed new rare medical conditions described as

being admirable and monstrous.[53] This was later followed by a description and drawings of a post mortem dissection by Fredericus Ruysch in 1670.[54]

Early attempts by physicians to treat BE surgically, and to restore urinary function have been sparse and notably unsuccessful. The first urine collector was designed in 1780[55] and from the mid-1850s and onwards, surgical approaches were undertaken for ureterosigmoidostomy, transplantation of the trigonum to the rectum and coverage, as well as augmentation of the bladder. However, all of these procedures were associated with severe long-term complications. In a Swedish context, Bergenhem was the first to describe BE in 1895 within an ethical surgical setting. He was also one of the first to perform the surgical technique of transplanting the ureters to the colon.[56] One also had to consider the length of anesthesia, which would have been fatal in this patient group until the introduction of endotracheal intubation in 1945. The continence rate after total reconstruction in the 1970s was reported as being as low as 20%, but with modern techniques it is today as high as 80%.[51]

Bilateral sacroiliac osteotomy was introduced by Trendelenburg as surgery with fixation of the pelvic sling and closure of the bladder plate in the first part of the 19th century. This did not come without complications and therefore the invention of a device called “the pelvic sling”, used for immobilization of the patient and approximation of the pubic bones came into practice.[51,57]

1.4.2 Developmental malformations of the urinary bladder and the abdominal wall

1.4.2.1 Bladder exstrophy

BE is a rare congenital malformation affecting both males and females. The prevalence of BE has been reported to range between 1.6 and 4.0 per 100 000 live births and with male-to female ratios between 1.0 and 2.8:1 in different regions.[58-67]

The child presents with a defect of the lower abdominal wall, which exposes an open bladder. The malformation also involves the urethra, genitalia, and pelvis, with separation of the pubic bones and the *rectus abdominis* muscle. The umbilicus is situated lower and the anus is ventrally displaced. Boys present with epispadias, with the urethra presenting on top of a flattened penis, and girls with a cleft clitoris and a shorter vagina (Figures 8 and 9). The spectrum of genitourinary malformations ranges in severity from epispadias, affecting only the urethra, and BE to cloacal exstrophy (CE), also involving the intestines. BE is the most

common form of this bladder exstrophy-epispadias-complex (BEEC) while epispadias and CE are reported with approximately the same prevalence, ranging between 1-2:100 000 with CE being slightly rarer.[68-71] BE is commonly considered to be an isolated malformation,[58,63,72] but concomitant malformations, such as cleft lip-palate, spina bifida, orthopedic, and gastrointestinal anomalies, have also been described.[61,64-65,73]

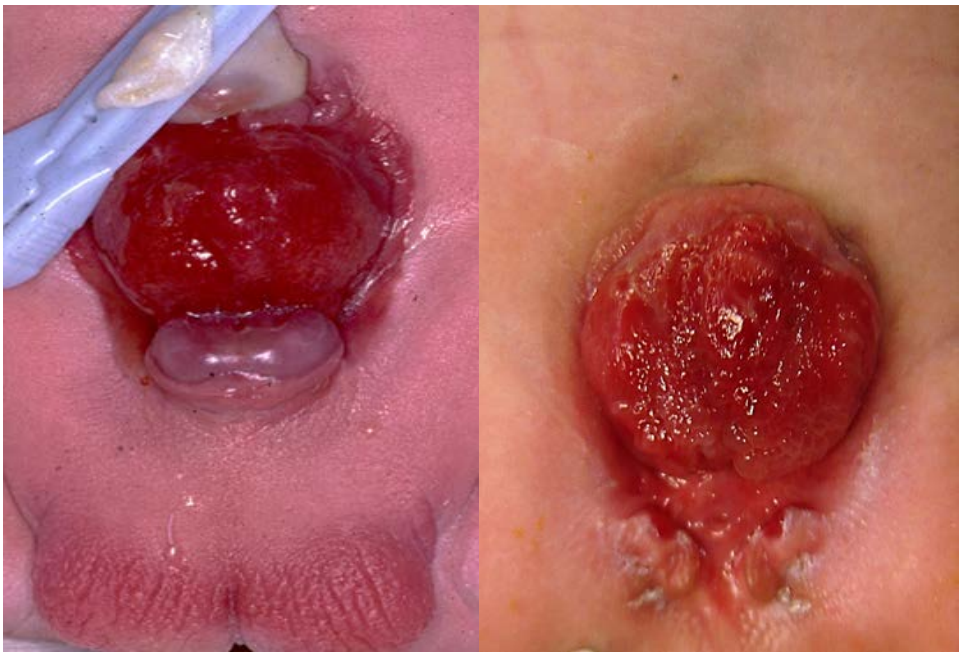

Figure 8. Photos of BE in a boy at birth (left) and a girl at primary surgery, two months old (right).

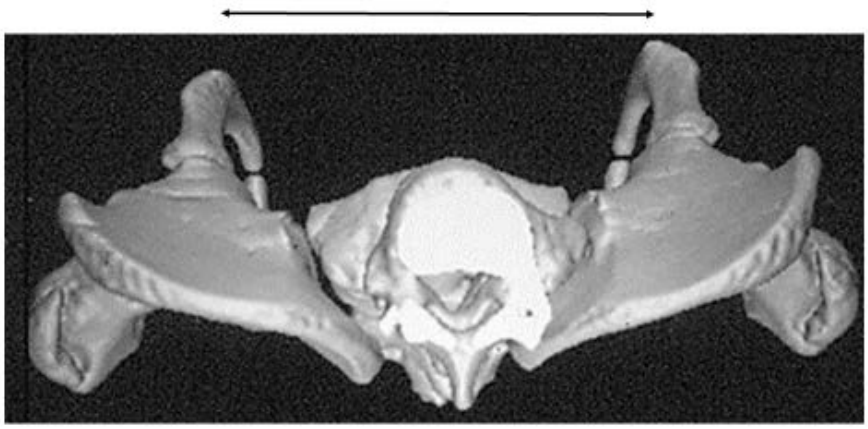

Figure 9. Pelvis from a nonoperated BE patient. Arrow indicates the widening of the pubic bone. Picture by courtesy of Bertil Romanus.

1.4.2.2 Abdominal wall defects

BE is sometimes described in a wider complex called OEIS where the child presents with omphalocele, BE, imperforated anus, and spina bifida, often also called CE. Due to the rarity of the disease, there are no large series that describe the prenatal spectrum of this condition or additional malformations.[74]

Other rare forms of abdominal wall defects are pentalogy of Cantrell, prune-belly syndrome and body stalk anomaly. In addition to these rare congenital abdominal wall defects, there are the more common malformations of gastroschisis and isolated omphalocele.[75]

1.4.3 Causes of developmental malformations in bladder exstrophy

The complex of epispadias, BE and CE are identified most often as having the same embryological and anatomical origin. The time of failure in development would then determine the severity of the malformation, the earlier the time, the more severe the malformation.[76] BE is a multifactorial malformation due to both genetic and environmental factors. The risk in siblings and offspring of BEEC patients is known to be increased to approximately 1:100 for siblings and 1:70 in offspring compared to 1:300 000 in the general population.[77-78] Monozygotic twins have also been shown to be more prone (62%) to be concordant for BEEC than dizygotic twins (11%).[78] This suggests the involvement of genetic factors in the etiology of BE.

1.4.3.1 Signaling pathways of the developing body wall and bladder

The pathogenic mechanism for the development of BE is not yet fully understood. Currently, it is mostly described as a failure of ingrowth of mesoderm, which may be due to early rupture of the cloacal membrane. It is interpreted as a failure of the lateral body wall folds to close in the midline of the pelvic region. During the 4th week, the sides of the embryo begin to grow ventrally, thereby forming two lateral body wall folds. These folds consist of mesoderm and migrating cells from somites. By the end of the 4th week, the lateral body wall folds normally meet in the midline and fuse to close the ventral body wall. Thus, one or both of the lateral body wall folds fail to progress ventrally or there are abnormalities in the fusion process once they meet in the midline. This could be due to either mechanical disruption or to enlargement of the cloacal membrane preventing the invasion of mesodermal cells.[79-81]

The epithelial-mesenchymal interaction is essential in bladder detrusor (smooth muscle) development. Bladder detrusor development, in the 12th week of gestation, requires a signal from the bladder urothelium, which is responsible for inducing mesenchymal development. The mesenchyme then further develops into the detrusor muscle.[82] Sonic hedgehog (Shh) has been identified as being involved in bladder development, as well as development of the external genitalia.[83-84] Failure of induction results in BEEC through a complex cascade of transcriptional factors affected by insertion or deletion.

1.4.3.2 Associated chromosomal aberrations

Over the years, there have been several reports on chromosomal anomalies in patients with BEEC. With better techniques that detect submicroscopic chromosomal aberrations, we have ascertained that the only recurrent aberration so far is the 22q11.2 microduplication. It is now known that the 22q11.2 microduplication is detected in 3% of all BE patients. This syndrome is also associated with hearing loss and neuropsychiatric symptoms; thus, an early diagnosis increases the opportunity to intervene to improve health care.[85-87]

1.4.3.3 Candidate genes for BE pathogenesis

WNT3

The *WNT* gene family encodes several glycoproteins that activate cell surface receptors involved in different signaling pathways. In this way, the WNT proteins are involved in multiple intracellular functions that are critical for many developmental processes, among others, organogenesis and organization of the body plan during embryonic development, as has been demonstrated in animal models.[88] Our group has identified a *de novo* mutation in the *WNT3* gene. In addition, expression of this mutant form of the WNT3 gene in zebrafish resulted in a malformed cloaca. Several other coding changes were identified in other *WNT* genes in BE patients but with more unclear significance.[89]

p63

P63, a tumor suppressor protein, is one of the regulators in epithelial stratification and is expressed in the urothelium of the ventral bladder. It can be found in the basal layer of the urothelium and also in the intermediate cells. *P63* has been identified as being involved in epithelial stratification, terminal differentiation, cell proliferation, cell-cell adhesion, and epithelial-mesenchymal signaling. When p63 does not work properly, or is absent, it

influences the transcriptional factors. Lack of epithelial signaling required for differentiation results in a lack of mesenchymal induction and subsequently failure of detrusor (smooth muscle) development. *P63* is expressed in all epithelia including the skin overlying the abdominal wall and the external genitalia.[90-94]

P63 is expressed in the urothelium throughout bladder organogenesis and is responsible for urothelium survival. Without an isoform of fetal *p63*, the ventral bladder epithelium is neither stratified nor differentiated in mice models, and subsequently undergoes apoptosis. The absence of *p63* has also been shown to result in reduction of the ventral ectoderm, which differentiates to form the epidermis, the skin, among other structures.[91, 95-96]

In conclusion, loss of *p63* expression in the ventral bladder leads to a decrease in cell proliferation of the bladder epithelium, loss of smooth muscle development due to a lack of mesenchymal induction, and a lack of stratified epithelial skin. This course of events may then result in ventral midline defects such as BE.

Perp

Another possible pathway, or perhaps a linked pathway, is via *perp*, which is closely linked with *p63*, but is also in a close relationship with the desmosomes, which are essential for maintaining cellular adhesion. *Perp* is also required for maintaining the integrity of the developing epithelium during organogenesis and is present in all embryonic mesenchyme surrounding the urogenital sinus and, lately, in all stratified epithelia. In overexpression, which may be the response to the absence of *p63*, cell adhesion becomes defected in the urothelium, as well as in epithelial skin.[96-98]

Disruption of the pathway of *p63* or *perp* may increase the risk of BE. In animals, genetic models of defects in *p63-perp* also develop the associated anomalies of BE.[96]

Pelvic development in BE

The above discussed hypotheses concerning the development of BE are mainly focused on the failure of development of the body wall and the bladder. They do not take account of the fact that failure of pelvic development with the bony structures could be involved. There are a few studies on pelvic development that suggest that the development of the urinary tract and the pelvic features are tightly connected: for example, the severity of the visceral features seem to be linked to the severity of the lack of rotation in the pelvic ring.[99-100]

In an animal model with chick embryos CE was identified and associated with abnormal aneurysmatic swellings of the dorsal aorta, resulting in distention of the developing pelvis and of the infra-umbilical portion of the developing body wall. Eventually, this part then became so thin that it ruptured.[101]

1.4.3.4 Environmental risk factors

Data on environmental risk factors are limited and inconclusive, probably due to the rarity of BE.[58,62,102] Maternal smoking has been reported to be an associated risk factor in CE[103] as well as high parity.[58,104] Obesity (BMI > 30) has also been identified as a potential risk factor for epispadias, as well as several other congenital malformations.[105] The pathogenic mechanisms of congenital malformations in obesity have been debated, in which connection, malnutrition, due to an inadequate diet and insufficient levels of essential micronutrients, may be involved. Similarly, maternal age has been recognized as a potential risk factor for BE, both for mothers <20 years of age[58] and for mothers of advanced age.[65]

1.4.4 Assisted reproductive technology and prenatal detection

1.4.4.1 Assisted reproductive technology

Assisted reproductive technology (ART) with *in vitro* fertilization (IVF) has been available for the treatment of infertility since 1977, with the birth of a baby girl in July, 1978.[106] Lately, it has been reported that ART is associated with an increased risk of birth defects in general, and also an increased risk for the development of BE.[107-109] But this has yet to be verified.

1.4.4.2 Prenatal detection

Since the fetal bladder can be detected on prenatal ultrasound from the 14th week of gestation, it is possible to demonstrate BE on a prenatal ultrasonography. Five criteria associated with BE have been identified: 1) a bladder never demonstrated on ultrasound; 2) a lower abdominal bulge representing the exstrophied bladder; 3) a diminutive penis with anteriorly displaced scrotum in males; 4) a low set umbilical insertion, and 5) abnormal widening of the iliac crests. However, on a retrospective review of selected prenatal ultrasounds conducted, only 67% of the cases were identified following the above criteria and 13% of these were actually made prior to delivery. The detection rate is estimated to be somewhat higher for male than for female fetuses due to a non-detectable urethra in males.[110-112] Parents will be offered prenatal counseling in case of antenatal detection of BE, which

may result in termination of the pregnancy. Still only approximately a quarter of the babies born with BE/CE are diagnosed antenatally. It is worth noting that not all diagnoses involving prenatal suspicion of BE are correct, thus an antenatal diagnosis may not be reliable.[113] Ultimately, if there is a decision to terminate the pregnancy, this would lead to a reduced prevalence and a possible shift in the sex ratio.

1.5 MANAGING BLADDER EXSTROPHY

1.5.1 Primary management

1.5.1.1 Management at birth

Since the bladder mucosa is exposed at birth, the umbilical cord should be carefully tied with a suture, rather than clamped, in order to prevent trauma to the mucosa. In addition, the bladder should be covered to prevent further trauma to the bladder mucosa.

1.5.1.2 Primary repair

Many different surgical procedures have been described for dealing with BE throughout history. Modern therapy aims at surgical reconstruction of the bladder and genitalia, most often through a staged approach. Today, there are two main approaches, but there is no uniform consensus on the timing of the surgery, and whether or not to perform an osteotomy simultaneously.

- Modern Staged Repair of Exstrophy (MSRE): the initial step is closure of the bladder, posterior urethral closure in boys and complete urethral closure in girls, and closure of the abdominal wall, with or without a pelvic osteotomy.
- Complete Primary Repair of Exstrophy (CPRE): the bladder closure is combined with epispadias repair, taking into account the size of the bladder and the penis in boys.

The reconstruction may take place within 72 hours of birth, under the influence of maternal relaxin, mostly if osteotomy is not opted for due to better compliance with tissue at this stage, or one to two months after birth when structures have stabilized.

1.5.1.3 Epispadias repair

This repair occurs around 6-12 months of age. The time and the extent of the surgery are dependent on the size of the bladder and the deformity of the penis. At this stage, the urethra, presenting on top of the flattened penis, is closed and transferred to below the

corporal bodies as in the normal penis, i.e. urethroplasty. This often results in hypospadias when the position of the urethral opening is situated on the ventral part of the penis. The urethra is often short and, sometimes, different flaps or grafts need to be used in stage repairs.

1.5.1.4 Urinary incontinence surgery

The size of the bladder gradually increases over time, sometimes with the use of a bulking agent in the bladder neck to increase bladder pressure and thus force the bladder to increase in volume.

Reconstruction of the bladder neck is typically done at toilet training age for BE patients, approximately age four to five years, when the child also shows interest in participating in a bladder retraining program. The timing of the surgery is also dependent on the capacity of the bladder. At surgery, the control of urine leakage is most often repaired with bladder neck plasty (BNP) and further enlargement of the bladder may be performed.

1.5.1.5 Female gynecology surgery

Female BE patients sometimes require surgery for vaginal and uterine prolapse in adulthood by means of different surgical techniques with hysterectomy sometimes being the only permanent option. The incidence of pelvic organ prolapses requiring surgical repair has been found to be 38%.[114] The onset of these conditions occurs in adolescence, but some of them only present during or after pregnancy.[115] Most pregnant women with BE undergo a caesarean section in order to minimize damage to genitourinary structures.[116]

1.5.2 Complications in bladder exstrophy

Worldwide historical and current reports on BE patients in the Third World describe different outcomes of patients born with BE. Some suffer from complications due to infection, electrolyte imbalance, and development of cancer, while others survive to an advanced age with or without surgery. Most of the patients in the Third World still present late in life when they survive childhood. Even with successful surgery, wound rupture is not uncommon and the patients may have long-term complications including urinary incontinence, reflux, bladder spasm, stone formation, and urinary tract infections.[117]

1.5.2.1 Urinary incontinence

Loss of voluntary control over the external sphincter muscle of the urethra causes urinary incontinence. Since the pelvic floor and muscles are abnormal in BE, this will be one of the major problems. The sphincter may be both abnormally developed as well as shortened. However, the condition may vary since the widening of the pubic bone and hence the impact on the pelvic floor structures, is individual. Voluntary bladder control is expected around the age of two to three years in the modern Western part of the world. The internal involuntary sphincter, relaxing on response to pressure, is non-existing at birth with BE since the midline is not closed. This sphincter may be underdeveloped or might be iatrogenically destroyed during primary surgery with closing of the bladder. Evaluation of the sphincters and, subsequently, future incontinence is not possible at the time of surgery, but there needs to be continuous evaluation during childhood. There is an ongoing debate over timing and type of surgery, and incontinence.

Unfortunately, there is no uniform definition of incontinence. The International Continence Society (ICS) recommendations state that urinary incontinence is a storage symptom and is defined as the complaint of continuous involuntary loss of urine.[118] This definition does not, however, take into account frequency, severity, duration, or impact. Most clinicians would probably assert that continence is defined by no leakage of urine during a period of at least two and up to three hours.

Several surgical techniques are described, or used when dealing with incontinence, for example bladder neck surgery, injection of a bulking agent, sling, implantation of an artificial urinary sphincter, closing of the urethral outlet or autologous cell injection.[119-121] None of these techniques are 100% successful and a combination of different procedures is often advocated. When performing surgery for incontinence one must also remember that this may also increase the pressure inside the bladder, resulting in reflux and the risk of kidney damage.

To avoid incontinence, self-catheterization of the bladder may be a good option. This can be done either through the normal urethra or, if the urethral outlet is surgically closed, through a stoma. The stoma is usually made out of the appendix, a Mitrofanoff stoma, but it can also be established by reconstruction from the small intestine, as a Monti tube.[122-124]

Incontinence in the BE population has been difficult to assess, but it seems that the most important criterion for improving the long-term prognosis is success of the primary closure.[125-126] It is now estimated that approximately 80-90 % of children with BE will be continent due to the improvement in surgical techniques.[127]

1.5.2.2 Ureterosigmoidostomy

The two main methods for replacing bladder function involve redirecting urinary flow (ureterosigmoidostomy or pouch), and enlargement of the bladder (augmentation).

Ureterosigmoidostomy was undertaken as early as in the 1800s (Bergenhem) with urinary diversion of the ureters to a distal colic segment. The main problem with this method was the effect when mixing urine and stool.

The risk of developing cancer after ureterosigmoidostomy has been determined to be 6-29%. The etiology is not fully known but it may be the result of bacterial action or from inflammation when urine and stool mix.[128-129]

During the last 30 years, bladder augmentation has largely been favored and replaced ureterosigmoidostomy in an attempt to overcome the risk of cancer and infection developing.[130]

1.5.2.3 Augmentation

Augmentations to restore bladder function and to prevent incontinence have been performed for over 100 years. Up to 70% of BE children need bladder augmentation or replacement to prevent incontinence.[127]
Several different intestinal and gastric segments have been used. They come with different pros and cons. Using a gastric segment is appealing because of the natural spherical configuration of the patch of the stomach used, in contrast to other intestinal segments, which have to be detubularized.[131] Surgery using a gastric segment is, however, more complicated due to mobilization of the segment and therefore, currently, mostly intestinal segments are being used. However, intestinal tissues are designed to absorb specific solutes, whereas bladder tissue is designed for the excretion of these same solutes. As a result, when gastrointestinal tissue is placed within the urinary tract, multiple complications may ensue. These include infection, metabolic disturbances, stone formation, perforation, increased mucus production, and malignancy.[131-135]

1.5.2.4 Other urinary diversions

There are several other approaches to the diversion of urine, for example, ileal conduit urinary diversion, an Indiana pouch reservoir, intestinal neobladder to urethra diversion and the Kock pouch. The method of choice is dependent on patient compliance, patient autonomy, comorbidity, and available health care standards.[136]

1.5.2.5 Comorbidity

Concomitant major malformations, such as cleft lip-palate, spina bifida, orthopedic and gastrointestinal anomalies, have been described[61,64-65,73] but BE mostly occurs as an isolated malformation.[58,63,72] As discussed previously, many of the known associated major malformations are also due to abnormal epithelial development or ventral midline defects. Many of the BE patients also require surgery for congenital inguinal hernia, as well as undescended testis. Congenital inguinal hernias have been reported to be more common among BE patients due to large internal and external inguinal rings and lack of obliquity of the inguinal canal.[137] The incidence of inguinal hernias among BE patients in the literature ranges from 56%-82% in males and 11%-15% in females.[138-139]

Psychiatric disorders

Psychiatric disorders have been discussed and highlighted in the BE population. Whether this would be a feature of the malformation spectrum or is due to psychosocial environmental factors is not evident. A few reports have identified more psychiatric disorders[140-142] and even suicidal ideation[143] in the BE population than in the general population while others have reported that the lower mental health reported in BE patients is not anywhere near that of the psychiatric patients.[144]

Hearing disorders

A few unrelated cases of BE carrying a microduplication of 22q11.2, which is associated with hearing impairment, have been identified. In several studies, the microduplication has now been identified in about 3% of all BE cases. The risk for BE is 50 times higher in carriers of this duplication.[85-87]

1.5.2.6 Fertility and pregnancy

Fertility is often stated as impaired in BE patients due to anatomical and physiological disadvantages, with males being more affected than females.[114-115,137,145-149]

A few studies have highlighted the difficulty to conceive naturally, while others have indicated no difficulties for BE women to get pregnant and conceive naturally, due to a possible favorable anatomical position of the uterus. Miscarriage in BE women has been reported to be as high as 18-35% and there are also reports on complications during or after pregnancy, and therefore pregnancy in BE is regarded as a high risk for both mother and offspring. Most pregnant women with BE undergo a caesarean section in order to minimize damage to genitourinary structures.[116,137,141,150-151]

1.5.3 Cancer of the urinary bladder and carcinogens

Generally, there is increasing evidence that the exstrophied bladder is at a significantly higher risk for developing bladder cancer, however, the pathogenic mechanism of the development of bladder tumors is not well understood. Bladder neoplasia may be due to either one or a combination of several mechanisms: the augmented segment may be deprived of its normal nutritional supplies, the direct toxic effect of the urine on the augmented segment, chronic bacteriuria causing toxic agents which could induce DNA mutagenesis, cell signaling between urothelial and augmented tissue, carcinogens as well as trauma, for example caused by clean intermittent catheterization (CIC), may be triggers. There is however, no evidence of significant correlation between chronic bacteriuria and bladder cancer, although this is generally thought to be the case. Cell signaling and an altered capacity of the exstrophied bladder to induce DNA mutagenesis, in and of itself, has been proven, but to what extent this causes bladder cancer has not yet been documented.[127,152-158]

The first papers published describing the occurrence of carcinomas developing after ureterocolic anastomoses appeared in the 1960s.[51] So far, the incidence of bladder cancer after augmentation in congenital malformations is unknown. Most bladder cancer in BE patients occur in patients who has never undergone reconstructive surgery.[157] However, tumors in BE patients in the Western World are seen mostly in patients with an augmented bladder. The surgical introduction of early closure seems to have had a positive effect on the bladder mucosa.[130,159] The tumors are predominantly adenocarcinomas (91-95%), in both augmented and neoexstrophied bladders.[51,157] Tumors of the bladder mainly arise in the 4th to 5th decade of life. The incidence in the general population is said to be about 0.02% overall

and, in the 6th decade of life, it is reported to be approximately 0.7%, but, in BE, it is reported to be as high as 4-7.5% throughout life which gives an increased risk of 7-235 times.[51,156]

Augmentation may be achieved by using either ileal or colonic tissue or by using gastric tissue. The increased risk of cancer may be 7-8 fold for ileal or colonic augmentation and 14-15 fold following gastric augmentation.[160] The normal incidence of cancer in the colon in the 6th decade of life is 5-6% while the incidence of the development of cancer in the ileum is only approximately 0.1%, which gives about a 40-50 fold increased risk when using ileal segments for augmentation.[155,158,160] The risk of gastric cancer in the general population in the US by the 6th decade of life is 0.3%, which gives a 30-40 fold increased risk for the gastric augmented bladder.[160] These data supports the procedure of using colonic segments when performing augmentation of the bladder to eliminate the otherwise increased risk of neoplasia.

1.5.4 The Swedish national surveillance program for bladder exstrophy

In Sweden, there has been a national surveillance program for BE since 2013. Before this, the four different pediatric surgery clinics in Sweden usually treated BE in the same way as now, and often discussed patients when surgery was forthcoming, in the same way as today. The surveillance program is a basis for surgery and follow-ups (Figure 10).

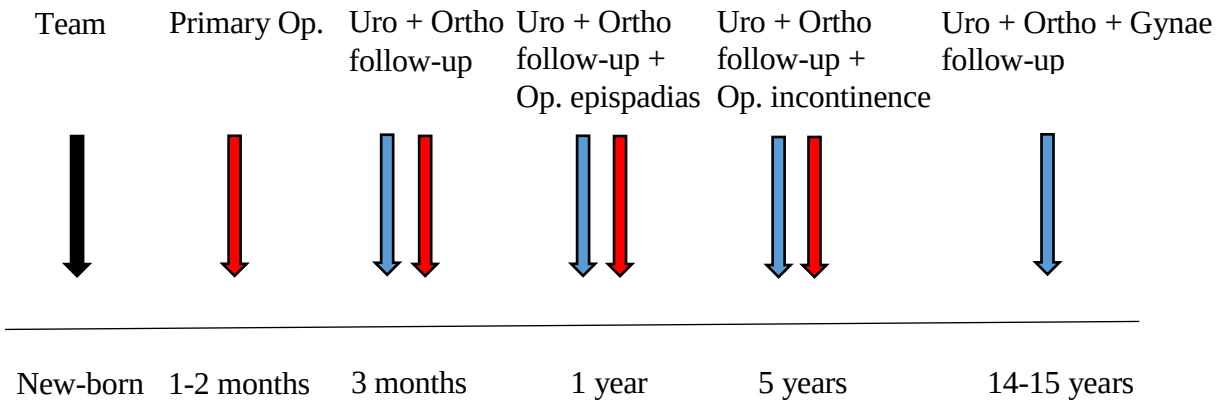

Figure 10. The Swedish national surveillance program for BE. Arrows indicating surgery in red and follow-up in blue. Adapted from the Swedish Healthcare Program for Bladder Exstrophy.

The time line presents the recommendation for a surveillance program but, it is, of course, adapted on an individual basis.

Echocardiography is mandatory at birth in order to exclude associated anomalies of the heart. Ultrasonography of the urinary system and radiology of the pelvis is performed at birth, and at 3 months, 1 year, 5 years and at approximately age 15 when the patient is referred to an adult urologist and/or gynecologist. Four-hours micturition is observed at 3 months, 1 year, 5 years and at approximately age 15. Incontinence, flow cystometry, and residual urine are evaluated at ages 5 and 15. Renal scintigraphy is evaluated at ages 1, 5 and 15. Cystometry or cystoscopy is performed at ages 5 and 15 to assess the volume of the bladder prior to incontinence surgery and referral to the adult team, and a voiding cystourethrogram is performed at 1 year. In girls, the genitalia are assessed under anesthesia at 15 years of age. The multi-disciplinary team consists of specially trained coordinating nurses, a pediatric urologist, a pediatric orthopedic surgeon, and a psychologist. Follow-ups and assessments are performed when necessary and are recommended on a yearly basis. Unfortunately, The Swedish national surveillance program for BE does not, include assessments and follow-ups in adulthood, but BE patients are referred to a specialized urological multi-disciplinary team consisting of a urologist and/or a gynecologist, a plastic surgeon, an urotherapist, and a psychologist.[161]

1.6 SWEDISH NATIONAL REGISTERS

Sweden qualifies as an exceptionally suitable country for register-based studies thanks to the existence of several nationwide population-based registers since 1952. The linkage of data from different registers is made possible by a unique 10-digit personal identification number (PIN) assigned to every legal resident of Sweden and is used in a wide variety of contexts, including health care and socioeconomic variables, making it possible to establish links between different registers. The PIN of a new-born is linked to the Medical Birth Register via The Birth Register at Statistics Sweden (SCB).[162]

Several different registers were used in Studies III and IV to identify the study subjects, outcomes, and exposures:

I. ***The Medical Birth Register*** (MBR) contains data on approximately 99% of all antenatal care and deliveries since 1973.

II. ***The Register of Congenital Malformations*** is formally a part of the MBR since 1973 and contains data since 1964. The registered data are based on reports of chromosomal

abnormalities and congenital malformations from maternity wards, pediatric wards, and cytogenetic laboratories. The register was expanded in 1999 to include malformations according to International Classification of Diseases (ICD) 10, including abortions due to malformations or chromosomal abnormalities. Dating from 2008, it includes all live births, independent of gestational week, and all births from the 23rd week of gestation, including stillborns.[163]

III. ***The National Patient Register*** **(**NPR) contains discharge diagnoses (primary and contributory) and surgery codes, according to the Swedish ICD and Classification of Surgical Procedures, covering hospital-based inpatient care since 1964, hospital-based outpatient care since 2001, and surgical day care procedures since 1997. Primary health care data are still not required to be reported on a national level to the Swedish National Board of Health and Welfare (SNBHW). In 1973, the NPR in-hospital care register covered 18 of 26 counties responsible for health care in Sweden and gradually finally covered all counties by 1987.[164]

IV. ***The Cause of Death Register*** contains data on all deaths of Swedish residents since 1952, coded according to the ICD system.

V. ***The Multi-Generation Register*** contains information about children and partnership between people born 1932 and registered from 1961 onward.

VI. ***The Register of Education*** holds information on education since 1985 and is updated annually with the highest obtained level of education for all residents from 16 years of age.

Registers *I-IV* are maintained by the SNBHW and *V-VI* by SCB.

The MBR, the NPR, the Multi-Generation Register and the Register of Education have all been externally evaluated. These evaluations confirm 85-95 % validity for the different registers.[164-168]

2 AIMS

The overall aims of this thesis include further investigation of the use of urothelium and the detrusor to form tissue *in vivo*, for the purpose of constructing a catheterizable conduit. Other aims were to increase current knowledge of BE and perhaps elucidate possible needs of future surgical applications of regenerative medicine.

The specific aims of the four studies were to:

Study I

- Transplant autologous minced tissue as a one-step procedure using the body as a bioreactor *in vivo* in a large animal model without cell culturing *in vitro*
- Determine if urothelial cells from minced bladder mucosa could migrate, reorganize and proliferate around a tubular structure *in vivo* in a large animal model, thereby creating a patent conduit of autologous urothelium, from the bladder to the skin to use for catheterization

Study II

- Determine if smooth muscle cells from minced detrusor could migrate, reorganize, and proliferate around a mould *in vivo* in a large animal model
- Determine if co-transplantation of minced urothelial mucosa and detrusor around a mould *in vivo* in a large animal model could strenghten a possible conduit for bladder catheterization

Study III

- Assess birth prevalence and perinatal characteristics of BE in Sweden over time via national population-based registers
- Analyze potential risk factors for BE in Sweden using national population-based registers

Study IV

- Analyze morbidity and mortality as well as the social parameters: partnership, biological children, and education in the BE poulation in Sweden, using national population-based registers

3 MATERIALS AND METHODS

3.1 STUDIES I AND II

These two studies were conducted in a large porcine animal model. Bladder tissue was obtained for studies on cell proliferation, migration, and reorganization on urothelium, as well as detrusor, smooth muscle cells, both *in vitro* and *in vivo*. Functional tests were performed by filling and emptying of the bladder and anatomical evaluations were performed macroscopically and with CT (Computed tomography) scans. The aim was to create a physically and anatomically functional catheterizable conduit from the bladder to the skin.

3.1.1 In vitro

In vitro analyses were carried out in Study II. Minced porcine detrusor muscle tissue particles were placed in a 24-well cell culture plastic and placed in a 37°C incubator with smooth muscle cell culture media for cell expansion *in vitro* according to the explant technique. Minced porcine urothelial tissue particles were handled in a similar way, but were cultured in urothelial cell culture media that was changed every other day, and at the same time cell cultures were assessed for viability and growth.

In order to study cell expansion in a 3D model that could be handled mechanically for tissue sectioning and histology, we used a cell-scaffold hybrid of collagen and a biodegradable nanopolymer, a polycaprolactone (PCL) mesh. Minced tissue was seeded on top of the scaffold and plastic compression, a method for reducing liquid, was performed incorporating the minced tissue into the scaffold. The scaffolds' upper surface was seeded with minced tissue consisting of detrusor only, detrusor and urothelium, or urothelium only, which was evenly and randomly spread, aiming for an overall 1:3 expansion. Scaffolds containing minced tissue were cut to fit in 12-well plates, placed in a urothelial cell culture medium, and cultured in a 37°C incubator. The medium was changed every other day and samples were analyzed after two or three weeks.

3.1.2 In vivo

Female Yorkshire-Swedish country pigs (11-12 weeks old) were used for the *in vivo* and *in vitro* studies. A midline incision below the umbilicus was performed in order to expose the urinary bladder and a lenticular area of the urinary bladder dome, measuring 12 cm longitudinally by 9 cm transversally (approximately ¼ of the total urinary bladder surface

area), was marked and removed. The detrusor muscle was separated mechanically from the bladder mucosa using surgical scissors and the two different tissues were minced separately with a mincing device producing particles measuring approximately 0.8x0.8 mm (Figure 11).

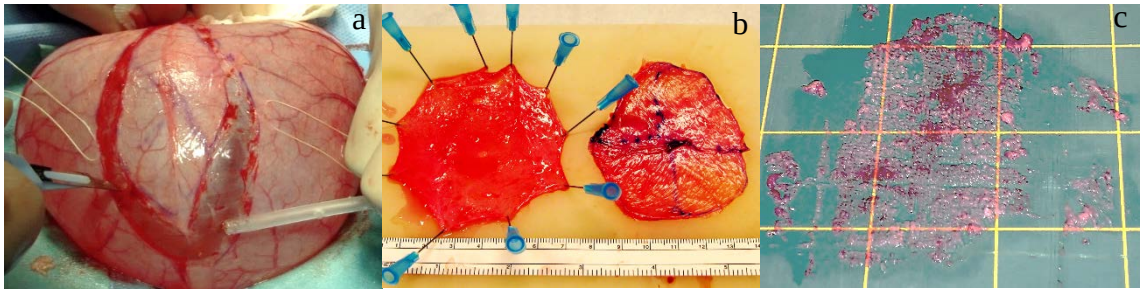

Figure 11. (a) Excision of bladder tissue. (b) Bladder mucosa mounted to the left and separated detrusor to the right. (c) Minced particles of bladder tissue. Adapted from Reinfeldt Engberg et al. Transplantation of autologous minced bladder mucosa for a one-step reconstruction of a tissue engineered bladder conduit. Biomed Res Int. 2013;2013:212734. Scientific paper I.

In Study I, aiming for a 3-fold expansion rate, we estimated the area of the inner surface of the tube used, by calculating the outer surface of a size 18 Fr and 10 cm long latex catheter, and dividing by three. Each end was closed with a 3 cm long segment of 16 Fr latex catheters sutured at right angles (stopping end) in order to secure the tubes in the bladder and the subcutaneous tissue.

For Study II, the appropriate amount of minced autologous tissue was calculated by measuring the outer surface of a size 16 Fr and 2 cm long latex catheter, sealed at each end, and dividing by three, aiming for the same 3-fold expansion of minced tissue.

The outer surface of the catheters in Studies I and II was covered with a two-component tissue sealant and pieces of minced tissue were applied evenly and randomly distributed. Moulds/tubes without minced particles were used as shams.

In Study I, each tube was placed in the bladder with a purse string suture around the end of the tube, and the tube was then inserted through the abdominal wall, before placement of the other end in the subcutaneous tissue, under the skin, and sutured to the tissue to stay in place. Thereafter, the wall of the urinary bladder and the abdomen were closed (Figure 12).

In Study II, the bladder was closed after taking of bladder tissue. Transcutaneous incisions lateral to the nipple ridge were performed down to the shivering muscle (*panniculus*

carnosus) and a subcutaneous space was created for horizontal placement of the cylindrical mould with the minced autologous tissue in the subcutaneous fat of the abdominal wall. The edges of the mould were secured to the underlying tissue and the subcutaneous fat was closed creating a tight-fitting pocket in order to minimize movement. A total of 23 moulds were transplanted. (Figure 12)

After the initial studies for Study I, surgery for Studies I and II were mostly undertaken at the same time and in the same pig using minced autologous tissue from the same bladder specimen. Animals were housed up to five weeks post transplantation.

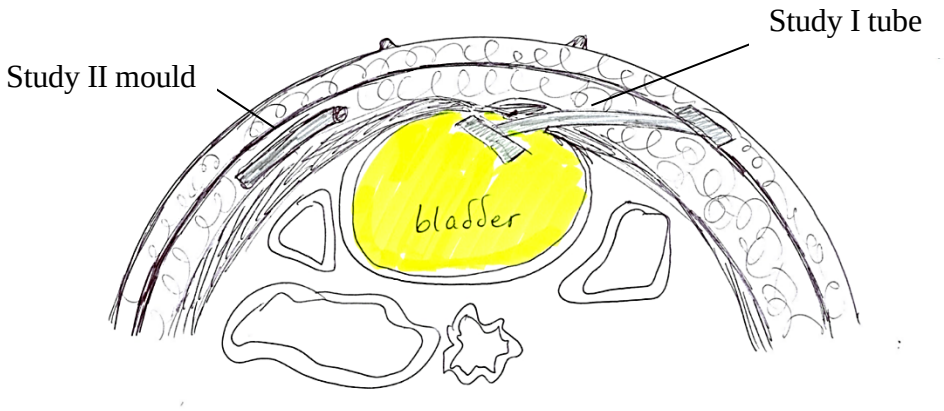

Figure 12. Drawing demonstrating a cross-section of the pig urinary bladder and abdominal wall with a tube placed to form a conduit from the urinary bladder, through the abdominal muscle wall and to the cutaneous tissue on the right (Study I) and a mould placed in the subcutaneous tissue on the shivering muscle on the left (Study II). Picture by Magdalena Fossum.

3.2 STUDIES III AND IV

These studies were conducted using national population-based registers between 1973 and 2011, Study III, and 1952 and 2011, Study IV. There were 213 available cases, Study III, and 365, Study IV, with a registered code of BE in the MBR with the Register of Congenital Malformations, the NPR and the Cause of Death Register. Study III was conducted as a matched case-control study while Study IV as a matched cohort study, both within the entire pool of live births in Sweden.

The inclusion criteria for Study III was being born in Sweden with a diagnosis of BE according to the ICD coding system between 1973 and 2011. Exclusion criterion were: not being diagnosed with validated codes for BE surgery or codes for comparable surgery or cystectomy, and accordingly no follow-up registries (Figure 13).

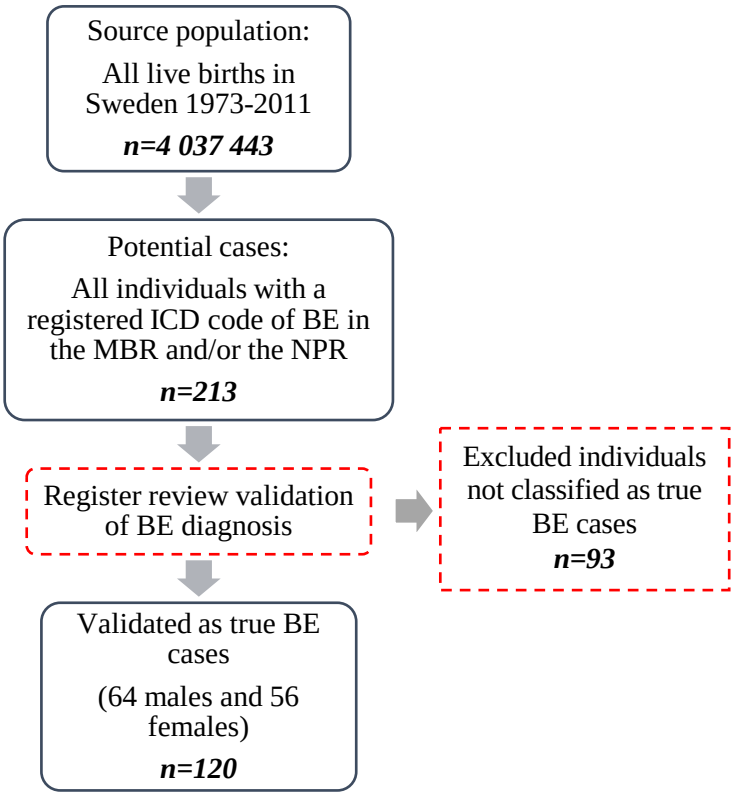

Figure 13. Flowchart displaying the identification of cases with BE between 1973 and 2011. Adapted from Reinfeldt Engberg et al. Maternal and fetal risk factors for bladder exstrophy: A nationwide Swedish case-control study. J Pediatr Urol. 2016 Jul 15. pii: S1477-5131(16)30135-8. Scientific paper III.

The inclusion criterion for Study IV was diagnosis of BE according to the ICD coding system between 1952 and 2011 without registration of emigration or immigration in the Multi-Generation Registry. Exclusion criterion were the same as for Study III (Figure 14).

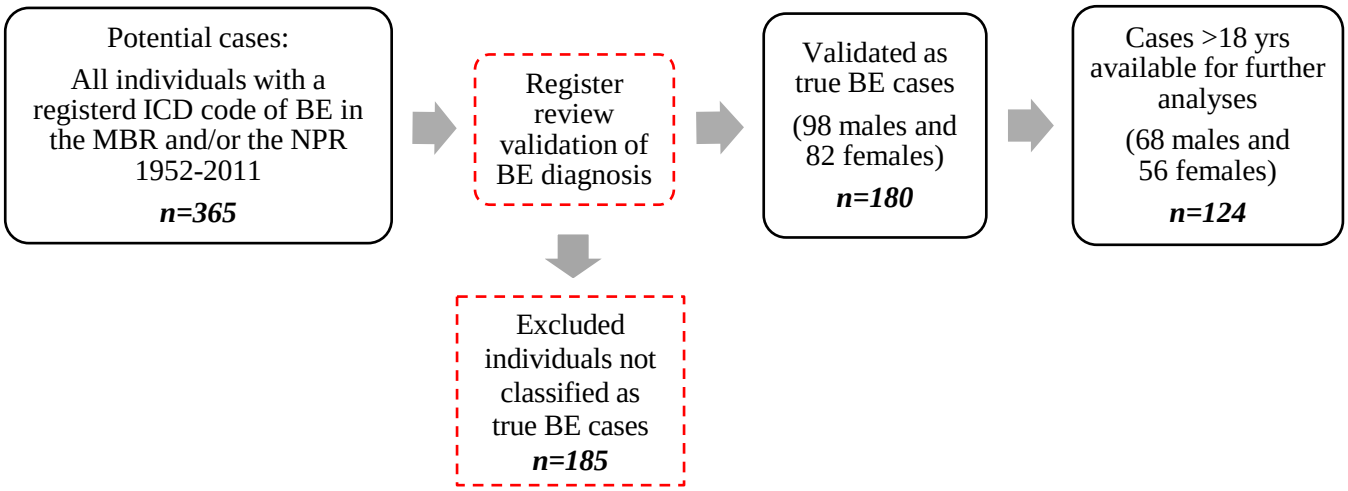

Figure 14. Flowchart displaying the identification of cases with BE between 1952 and 2011. Adapted from Scientific paper IV.

Several different national population-based registers were used in Studies III and IV to identify the study subjects, outcomes, and exposures. Prevalence, sex ratio, and birth descriptive data, including associated major malformations, congenital inguinal hernia, and undescended testis, were identified in Study III. Comorbidity and mortality (according to the ICD), partnership, biological children, and level of education were presented in Study IV.

3.2.1 Validation of cases

Cases and controls were identified within the population of total live births in Sweden between 1973 and 2011 for Study III, and between 1952 and 2011 for Study IV. Cases were subjects with a diagnostic code for BE; 757.3 [ICD-6], 757.3 [ICD-7], 753.50, [ICD-8], 753F [ICD-9] and Q64.1 [ICD-10]. Controls were matched by sex and age.

Three of the authors of Studies III and IV independently validated the available 365 individual cases with a diagnostic ICD code for BE between 1952 and 2011. The validation was carried out by excluding cases without registered codes for reconstruction of BE, codes validated as correct codes for reconstructive surgery on patients with BE or cystectomies as

primary surgery. In addition to surgery, available cases were also validated for number of follow-ups with the correct ICD code of BE in the NPR. 120 cases were validated as true BE cases for Study III, and 180 cases for Study IV.

Matching of cases was not done according to county, but an analysis of cases to county demonstrated no correlation of case distribution to county or to urban/rural areas.

3.2.1.1 Sensitivity analyses

Sensitivity analyses were carried out in both Studies III and IV.

In Study III the outcome, when including all available cases, were a slightly higher sex ratio with 1.54:1, male-to-female. The distribution was male cases 60.6% and female cases 39.4%, compared to analyses in the study of male cases 53.3% and female cases 46.7%, with a male-to-female ratio of 1.14:1. Further sensitivity analyses were not carried out for other variables because a validation of excluded cases revealed other diseases or anomalies that likely would have influenced the outcome of the sensitivity analyses. Furthermore, excluded cases included more males due to misclassification and incorrect coding, for example in cases diagnosed with epispadias only or posterior urethral valves. This was not as common in females as in males, i.e. none of the excluded females were diagnosed with epispadias.

In Study IV sensitivity analyses were only carried out for the outcome of number of males and females since neither the prevalence nor the sex ratio was an aim of this study. Within all available cases, males constituted 59.8%, and females 40.2% compared to males 54.4%, and females 45.6% validated as true BE cases in Study IV. Further sensitivity analyses were not undertaken because of diagnosis of other diseases or anomalies of the excluded cases, which might have influenced the sensitivity analyses, in the same way as in Study III.

In Study IV available cases with immigration and/or emigration were excluded due to lack of reliability of covering in the registers. These cases constituted only nine additional cases. However, these cases may be included in future analyses concerning surgery and psycho-social outcome in questionnaire or medical record based research.

3.3 STATISTICS

In Study III odds ratios (OR) were calculated with confidence interval (CI) 95% for univariate and multivariate analyses for BMI, age, and smoking to determine the risk for BE with each variable. Numerical continuous data were presented as numbers, means, and

proportions for both Studies III and IV while categorical data was presented as frequency counts and percentages for Study IV. Continuous data for Study IV were analyzed using *t*-test for independent samples and two-way analysis of variance (ANOVA) when comparing cases and controls adjusting for gender. Chi-square test or Fisher's exact test were used for categorical data. For Study IV, p-values <0.05 were considered statistically significant.

3.4 ETHICAL CONSIDERATIONS

3.4.1 Studies I and II

The protocols of Studies I and II were pre-approved by the Stockholm County Committee on Animals and all the procedures conformed with local regulations for the use of animals, as well as with the relevant national statutes, Ethical Permission C 152/9.

Research in animals constitutes a necessary model to secure a good practice and to refine protocols, tissue handling, and surgery prior to clinical trials on patients.

When performing scientific studies on animals it is important to consider the 3Rs: Replace, Reduce and Refine. These aspects are to ensure that 1) other possible methods of non-animal use have been considered for the study (Replace), 2) the lowest possible number of animals is being used in the study (Reduce), and 3) the choice of animal is correct for the study and the reason why this animal model is the most refined one, including methods of securing minimal suffering of the animals (Refine). This must be stated in the application form for ethical approval of the study.

A large animal model was chosen after ethical considerations for Studies I and II, using a porcine model. This was based on the pig being suitable for anatomical reasons due to a large urinary bladder, to which it is also easy to gain access. The urinary bladder had to be large enough to withstand a defect that yields enough tissue for the study, and therefore a smaller animal could not be considered due to their smaller urinary bladders. By these means, it was also possible to study anatomical and physiological characteristics comparable to the conditions in humans. It is not possible to conduct studies in regenerative medicine that introduce new surgical procedures *in vitro*, but to ensure the lowest possible number of animals being used, the studies were conducted with four animals at a time and stopping when sufficient results required for the study were obtained.

Strict protocols concerning the animals' well-being, including controls for urination pre- and post-operatively, signs of infections, surgical side effects and check-ups on dressings and wounds several times daily throughout the study period, were followed. Evaluations of the pigs' well-being were performed several times daily by a veterinarian who had the exclusive right to determine whether or not to administer supplemental medication or to terminate the pig if the animal showed signs of pain or distress. The pigs had a full supply of fresh water and were fed according to standard protocols at the animal institution where they were stalled. Animals were kept in pairs, except immediately post operatively, until they regained normal activity. After removal of the tissue transplants under general anesthesia, the animals were euthanized with a lethal dose of sodium thiopental.

3.4.2 Studies III and IV

These studies were approved by the Karolinska Institutet Ethics Committee before data requisition from the national registries, Ethical Permission 2011/1122-31/4, 2013/830-32, and 2013/2076-32.

The Swedish healthcare system is publicly funded and therefore ensures everyone equal access to health care services. A unique personal identification number (PIN) is assigned to all residents and can be used for linking national registers. The registers were linked by both the SNBHW and SCB for different periods of time. Cases were given a special serial number to be linked between the registries while maintaining patient anonymity. The key was kept by the SNBHW and SCB.

Since BE is a rare congenital malformation, we also had to consider patient anonymity when reporting on particularly mortality and morbidity outcomes in Studies III and IV. It was of special concern that it would not be possible to reveal the identity of any case based on the information in the studies and, in the case of rare specific comorbidities, we decided not to present detailed outcomes.

4 RESULTS

4.1 STUDY I

All pigs tolerated the excision and removal of bladder tissue well. The surgical technique had to be changed during the study due to the appearance of hernia in four animals and two animals showed signs of incarcerated small intestine and were therefore terminated. In addition, one animal was terminated due to perforated ulceration of the ventricle. As a result, a total of five animals were excluded due to surgical complications.

Conduits were assessed with respect to the macroscopic and microscopic appearance in six pigs, four with minced tissue and two shams. A gross examination revealed a patent conduit with an opening to the bladder. Histology and immunohistochemistry demonstrated a multilayered transitional uroepithelium in all four cases. Up to 89% of the luminal surface area was neoepithelialized but with a loose attachment to the submucosa. No epithelium was found in the control animals (Figure 15).

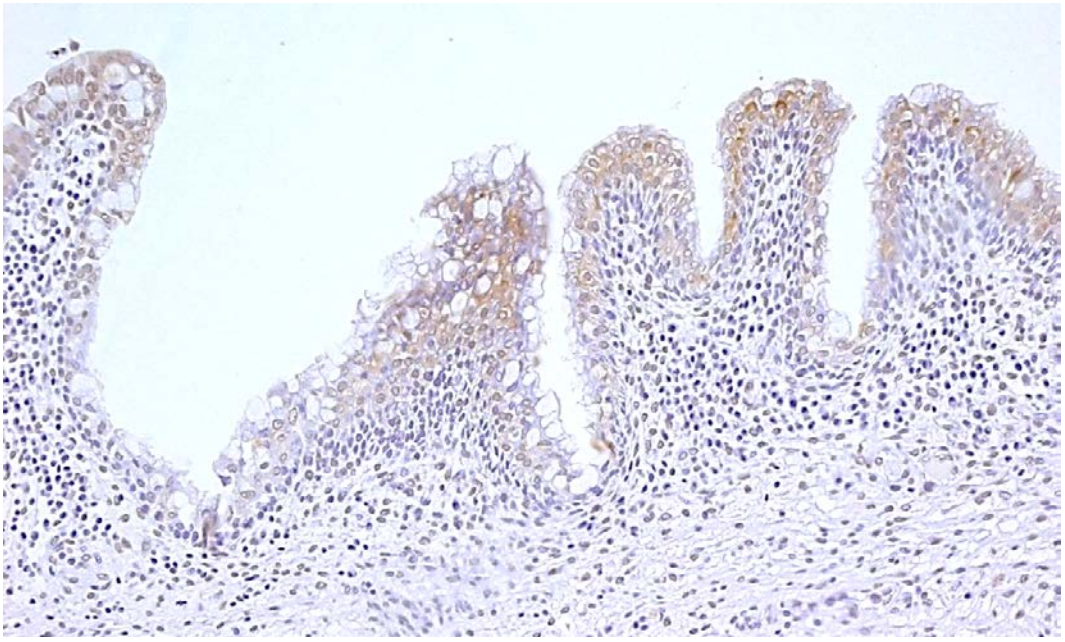

Figure 15. Photomicrograph demonstrating neourothelium in a neo-regenerated conduit. (Uroplakin III, x100). Adapted from Reinfeldt Engberg et al. Transplantation of autologous minced bladder mucosa for a one-step reconstruction of a tissue engineered bladder conduit. Biomed Res Int. 2013;2013:212734. Scientific paper I.

Two pigs underwent radiology before termination. CT imaging revealed a patent channel that could be used for filling and emptying of the bladder (Figure 16).

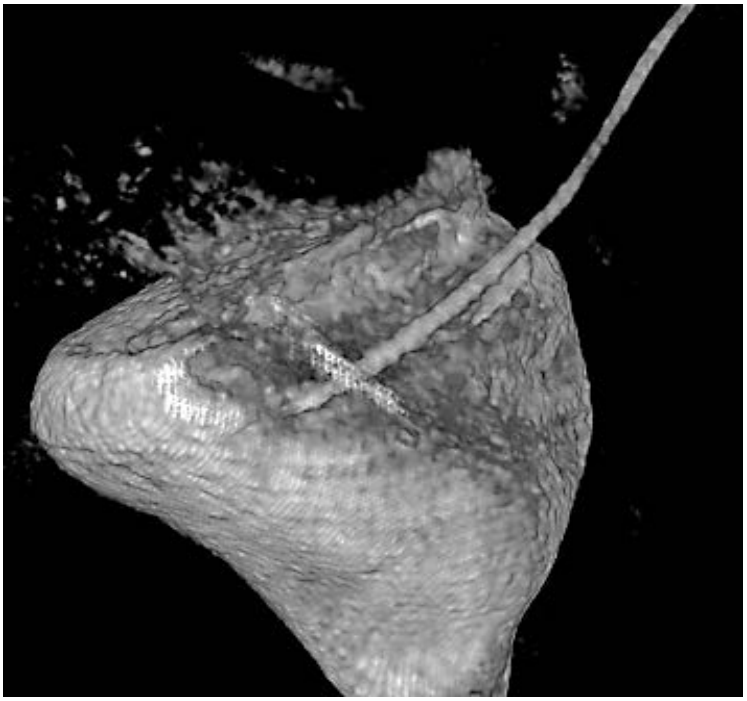

Figure 16. 3D reconstruction of the bladder demonstrating a patent conduit from the bladder to the skin, filled with contrast after removal of the tube. Reinfeldt Engberg et al. Transplantation of autologous minced bladder mucosa for a one-step reconstruction of a tissue engineered bladder conduit. Biomed Res Int. 2013;2013:212734. Scientific paper I.

4.2 STUDY II

In vitro

Cell expansion using the explant technique showed cell migration and regeneration from minced detrusor and urothelium with a morphology typical of smooth muscle and urothelial cells, respectively.

3D cell expansion on scaffolds showed cells of nonepithelial origin with a morphology of smooth muscle cells only in cultures that had been seeded with minced detrusor muscle. Immunohistochemistry and morphology confirmed that these were smooth muscle cells, staining for alpha-actin (Figure 17 a).

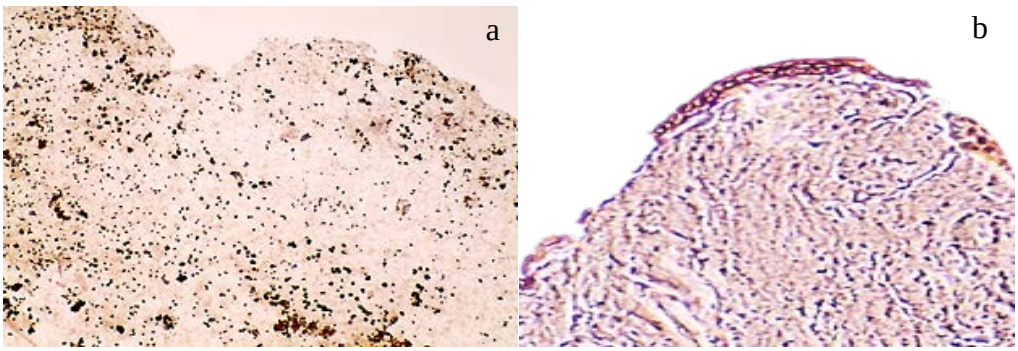

Figure 17. Photomicrograph demonstrating morphology of 3D cell expansion on collagen scaffolds. (a) Demonstrating smooth muscle cells (Alpha-actin, x100), and (b) the lining of urothelial cells (CkMNF116, x100). Adapted from Reinfeldt Engberg et al. Expansion of submucosal bladder wall tissue *in vitro* and *in vivo*. Biomed Res Int. 2016;2016:5415012. Scientific paper II.

Scaffolds with minced urothelium, alone or together with minced detrusor muscle, showed an epithelial lining on the surface and immunohistochemistry with cytokeratins confirmed the urothelial origin (Figure 17 b). The urothelium stained for Ki-67, indicating a sustained proliferative capacity.

In vivo

Detrusor regeneration and proliferation

Cells from minced detrusor muscle reorganized and proliferated in two out of five (40%) of the transplants with minced autologous detrusor only, and four out of six (67%) of the transplants with a minced autologous detrusor and urothelium. The specimens showing detrusor muscle proliferation, altogether six out of eleven (55%), showed continuous muscle tissue separated from the underlying shivering muscle and reorientated around the mould (Figure 18 a). No detrusor muscle was present in transplanted minced autologous urothelium only, nor in shams.

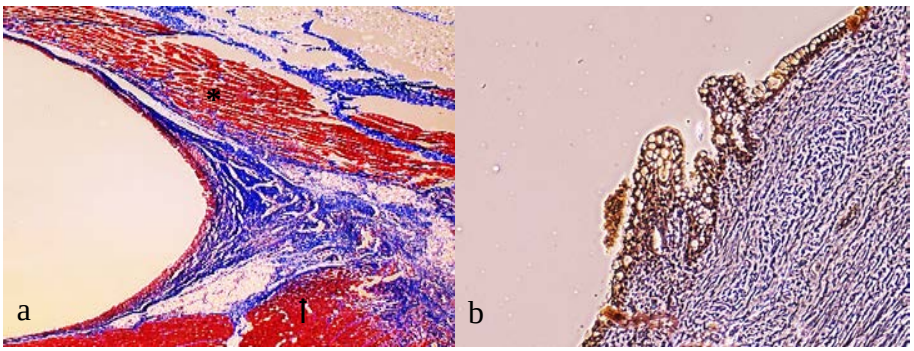

Figure 18. Photomicrograph of regenerated minced detrusor and urothelium. (a) Regenerated detrusor muscle indicated by (*), separated from the shivering muscle marked with (†) (Masson's trichrome, x50), and (b) a multi-layered continuous transitional epithelium of urothelial origin (Uroplakin III, x100). Adapted from Reinfeldt Engberg et al. Expansion of submucosal bladder wall tissue *in vitro* and *in vivo*. Biomed Res Int. 2016;2016:5415012. Scientific paper II.

Urothelial regeneration and proliferation

A continuous transitional cell epithelium covering the luminal surface and up to five cell layers thick was present in transplants with minced autologous urothelium only, but not in any of the moulds with co-transplanted minced autologous urothelium and detrusor muscle. Immunostaining with uroplakin III confirmed the presence of urothelium (Figure 18 b).

4.3 STUDY III

We identified 120 patients between 1973 and 2011 who were validated as true BE cases. The prevalence was calculated to be approximately 3:100 000 live births and was stable over time (Figure 19), with a male-to-female ratio of 1.14:1.

	N/ 100 000 LIVE BIRTHS (95% CI)		
YEARS	**All**	**Male**	**Female**
1973-1982	2.83 (1.78-3.87)	2.55 (1.16-3.94)	3.11 (1.54-4.69)
1983-1992	3.12 (2.07-4.17)	3.75 (2.14-5.35)	2.46 (1.12-3.79)
1993-2002	3.69 (2.49-4.90)	3.40 (1.78-5.01)	4.01 (2.20-5.81)
2003-2011	2.28 (1.33-3.24)	2.62 (1.20-4.05)	1.92 (0.67-3.18)

Figure 19. Decennial prevalence of BE between 1973 and 2011.

In 92.5% of the cases, BE was an isolated malformation without associated major malformations. However, 41% of all cases required surgery for congenital inguinal hernia

and 11% of the male subjects had had surgery for undescended testis. A significantly higher proportion of cases had a birth weight <1500 g, compared with controls, but other characteristics were comparable to those of controls.
The only potentially associated maternal risk factor was advanced age (Figure 20).

A.	**Cases** **N = 120**	**Controls** **N = 600**	
Infant, sex			
Male	64 (53.3 %)	320 (53.3 %)	
Female	56 (46.7 %)	280 (46.7 %)	
Birth weight (g), mean ± SD	3405 ± 617	3499 ± 563	
<1500	5 (4.2 %)	4 (0.7 %)	
	Cases **N = 120**	**Male** **N = 64**	**Female** **N = 56**
Associated major malformations	9 (7.5 %)	3 (4.7 %)	6 (10.7 %)
Inguinal hernia	49 (41.0 %)	40 (62.5 %)	9 (16.1 %)
Undescended testis		7 (11 %)	
B.	**OR (95% CI)[a]**		
BMI (obesity vs normal)	1.44 (0.57-3.63)		
Age (≥35 yrs vs 25-29.9 yrs)	3.60 (1.62-7.99)		
Smoking, at any time	0.98 (0.47-2.05)		

Figure 20. Summary table. **A.** Birth descriptive data on infants born with BE, and **B.** Multivariate logistic regression model of potential maternal risk factors. Model adjusted for maternal age >35 years, smoking at any time during pregnancy, and obesity. Table adapted from Reinfeldt Engberg et al. Maternal and fetal risk factors for bladder exstrophy: A nationwide Swedish case-control study. J Ped Urol. 2016 Jul 15. pii: S1477-5131(16)30135-8.

4.4 STUDY IV

Data on variables in Study IV are presented in Figure 21. Altogether, 180 cases were identified as true BE cases between 1952 and 2011, 98 (54.4%) male and 82 (45.6%) female cases, were available for descriptive analyses concerning mortality and morbidity, i.e. cancer, and psychiatric and hearing disorders. Among these 180 cases, 54 were under 18 years of age and two died in infancy, thus 124 cases > 18 years of age were available for further statistical analyses, 68 males (54.8%) and 56 females (45.2%).

The mortality and comorbidities involving bladder cancer, and psychiatric disorders were in line with those of controls and the general population, and hearing disorders of sensory neurogenic background were present in five, equaling 2.8% of the cases, which was in concordance with previous findings of possible genetic association of 3% of all BE cases with sensory neurogenic hearing loss and WNT3 gene mutation.

Registered partnership data were comparable to those of controls for both men and women. Cases, of both sexes, conceived significantly fewer biological children; however, the mean age for the first child was not different. The number of children per female BE patient differed significantly from that of comparable controls with no female case conceiving more than two children.

The overall educational level was high with slightly fewer male and slightly more female cases reaching the highest level compared to controls.

A. **1952-2011**	**Cases** **N = 180**	**Male** **N = 98 (54.4%)**	**Female** **N = 82 (45.6%)**
Mortality	9 (5.0%)	5 (5.1%)	4 (4.9%)
Bladder cancer	4 (2.2%)	2 (2.0%)	2 (2.4%)
Psychiatric disorders	13 (7.2%)	8 (8.2%)	5 (6.1%)
Hearing disorders	5 (2.8%)	3 (3.1%)	2 (2.4%)

B. **1952-1993,** **>18 yrs**	**Cases** **N = 124**	**Male** **N = 68** **(54.8%)**	**Female** **N = 56** **(45.2%)**	**Controls** **N = 620**	**Male** **N = 340** **(54.8%)**	**Female** **N = 280** **(45.2%)**	**p-value**
Partnership	52(41.9%)	26(38.2%)	26(46.4%)	244(39.4%)	111(32.6%)	133(47.5%)	0.59
Age 1st partnership, mean ± SD	30.6 ± 5.6	31.0 ± 4.7	30.2 ± 6.3	29.6 ± 6.2	31.5 ± 6.1	28.1 ± 6.0	0.30
Biol child	39(31.5%)	17(25.0%)	22(39.3%)	356(57.4%)	172(50.6%)	184(65.6%)	**<0.0001**
Age 1st biol child, mean ± SD	29.7 ± 6.0	32.0 ± 6.0	27.9 ± 5.4	28.4 ± 5.5	29.9 ± 5.0	26.9 ± 5.0	0.15
Dist of child							**0.0218**
1	16(41.0%)	6(35.3%)	10(45.5%)	88(24.7%)	48(27.9%)	40(21.7%)	M=0.86
2	20(51.3%)	8(47.1%)	12(54.6%)	170(47.8%)	78(45.4%)	92(50.0%)	**F=0.008**
3	3(7.7%)	3(17.7%)	0(0.0%)	73(20.5%)	35(20.4%)	38(20.7%)	
>3	0(0.0%)	0(0.0%)	0(0.0%)	25(7.0%)	11(6.4%)	14(7.6%)	
Education							0.75
Level 1-3	41(35.7%)	29(47.5%)	12(22.2%)	196(34.1%)	112(35.8%)	84(32.1%)	M=0.08
Level 4-7	74(64.4%)	32(52.2%)	42(77.8%)	379(65.9%)	201(64.2%)	178(67.9%)	F=0.15

Figure 21. Summary table. **A.** Descriptive data of BE cases between 1952 and 2011, and **B.** Logistic regression model and ANOVA between 1952 and 1993, >18 years of age. SD = standard deviation.

5 DISCUSSION

5.1 STUDIES I AND II

Both Studies I and II demonstrated the possibility of transplanting minced bladder tissue *in vivo*, using regenerative medicine techniques. In Study I, we could also demonstrate the possibility to create a conduit to the urinary bladder by randomly transplanting small particles of autologous urothelial tissue on a tube. The particles reorganized, migrated, and proliferated. It would be easy to perform the method as a one-step procedure in the operating room with no need to culture cells *in vitro*.

The urothelium was, however, loosely attached and could not withstand the repeated trauma of catheterization. In order to strengthen the conduit, we therefore co-transplanted minced detrusor muscle and urothelium, as described in Study II. Unfortunately, co-transplantation was not favored in the sense that the urothelium did not survive *in vivo* when transplanting it together with the detrusor muscle. This might be explained by the natural conditions in the transplantation environment. Transplants were located on the shivering muscle of the abdominal wall and thus detrusor muscle cells were in contact with other muscle cells making possible cross talk influence regeneration. On the other hand, the urothelial cells were not under the influence of other equivalent cells in the urinary tract. Nevertheless, transplanted minced urothelium alone did proliferate under these conditions. The cross talk, signaling systems, between cells might then be an explanation for the failure of co-transplantation. In the embryological development of the bladder wall, it has been ascertained that the urothelium develops long before the detrusor muscle where the development is induced by the urothelial cells. When co-transplanting urothelium and detrusor muscle, this condition is overruled, which might in turn lead to apoptosis or non-generation of urothelial cells. The reason for this scenario is not clear.

Further studies are needed to evaluate co-transplantation in order to strengthen a possible catheterizable bladder conduit. With further success, this could be useful for a large group of patients requiring bladder emptying by catheterization through a cutaneous stoma.

The pig is anatomically well suited for studies on the urinary bladder, however, they gain about one kg every day, which in turn put great stress on the surgical area. In the beginning of our studies. this even led to hernias, which affected the study results, and of course the

wellbeing of the animals. The dressing of the wounds was also very difficult to apply since all animals are very interested in investigating and biting off each other's dressings. Most of our pigs had t-shirts on for a few days after surgery. Maybe the pig is not the best animal after all for our studies. For future studies, the rabbit may also be an animal for consideration for bladder studies, although with a considerably smaller bladder. This would also be more ethical according to the 3Rs since the rabbit is considered to be of a lower status than the pig. Although, this, may not be of everybody's personal opinion, but nevertheless true in ethical considerations.

5.2 STUDY III

This study demonstrated a prevalence of BE in Sweden which is well in the range with that of other European countries and the United States. There was no change over longer periods of time, but there was marked yearly variation in prevalence, which also elucidates the importance of conducting long-term research when dealing with rare congenital malformations. The prevalence over time has been discussed lately in the context of prenatal ultrasonography detection. Some have argued that prevalence is on the decline due to advice concerning the termination of pregnancy when BE is detected. It is generally thought that boys with BE are more easily detected due to the fact that there is no apparent urethra and therefore a more thorough ultrasound examination is conducted. Ultimately, if there is a decision to terminate the pregnancy, this would lead to a reduced prevalence and a possible shift in the sex ratio.

We found an equal sex ratio, that is not usually presented in the literature. The reason for a skewed sex ratio in favor of the boys being more affected might be a manifestation of the fact that boys are more closely followed up due to more surgery during childhood. The girls are then lost to follow-up until adulthood and therefore are not present in the prevalence data. With regard to what has been discussed earlier in this thesis concerning prenatal detection, boys are easier to detect due to absence of a long urethra and, therefore more boys would be chosen subsequently for termination. In the future, this would then result in a more equal sex ratio. However, in our material prenatal detection has not been an issue because it has only been regarded as possible during the current decade.

Study III showed a significant difference in BE patients born with a gestational weight <1500g. However, on conducting a sub analysis, this turned out to be due to BE cases born with multiple associated malformations.

In Study III, significant statistics were difficult to obtain, most probably due to the small sample size and the wide range in confidence intervals. We only had information on 66 cases for BMI, and between 27 and 98 cases, dependent on the time period in the pregnancy, for smoking. When conducting research on rare congenital malformations, one has to settle with the available cases in contrast to calculating power in cases needed to treat. This will, in turn, be rather frustrating when discussing potential risk factors. In Study III, the only potential risk factor, after adjusting for BMI and smoking, was advanced age, which has been identified previously in other congenital malformations.

Smoking was the only potential risk factor that was possibly under the influence of bias since this was retrospectively reported approximately 10-12 weeks into the pregnancy.

5.3 STUDY IV

Study IV demonstrated a mortality rate in concordance with that of other European countries. The Swedish BE population did not show an increased risk of bladder cancer when compared with the general population. The risk of psychiatric disorders was also in concordance with that of the general population in Sweden, which is, in contrary to general beliefs, in the same range as for other European countries. Hearing disorders with a sensory neurogenic background were present in five cases, indicating a possibility of a genetic predisposition involving the 22q11.2 microduplication syndrome. Hearing disorders would be interesting to validate even further in other nationwide populations, and with pooling of more patients, thereby indicating a possible associated molecular background.

Stable partnerships where formed to the same extent and at the same time in life as in the general population in Sweden. This may indicate that BE patients in Sweden regard themselves as not being different from their peers of the same age and may also indicate a more positive self-perception than has previously been described in the literature. The identification of a generally high educational level is also positive with regard to assumed associated socioeconomic disadvantages for BE patients.

One of the most important findings in Study IV was the results regarding fertility. Both male and female cases gave birth to fewer children. No women with BE had more than two children, which might imply problems during pregnancy or after giving birth. This has not been established previously in a large unbiased study cohort. Whether this is due to the developmental malformation *per se* or to a combination with disadvantage after surgery remains to be analyzed.

More detailed future analyses are needed regarding socioeconomic conditions in adulthood. This could be undertaken with questionnaires dealing with psychosocial conditions and, clinical follow-up of the urogynecological status, as well as analyses of urinary incontinence and fertility. In addition, an evaluation of the medical records could be undertaken to determine the type of surgery.

It was unexpected that so many of the cases had to be excluded in Studies III and IV due to misclassification. The registers have been externally validated and are generally thought to be of good quality. The largest default of the registers is believed to be the lack of coding or incorrect coding.

In both Studies III and IV, national registers were used. The benefits of registers are numerous. Registers are anonymous, possibly nationwide, officially updated, and nationally supervised. Data may be used to detect differences over time both on a national, as well as, on a local level, and used as prognostic tools when assessed over longer time periods. However, one might argue that registers are monitoring your personal life and that it should not be mandatory to be registered but, then again if not mandatory, registers would lose validity as prognostic and official data.

6 CONCLUSIONS

6.1 STUDY I

- We demonstrate that minced urothelium can be transplanted in a random fashion on a tube placed in a surgically created canal through the abdominal wall. The urothelial cells will reorganize, migrate, and proliferate to form a continuous epithelial lining around the tube. Upon removing the tube, a well-defined conduit was present which could be used for catheterization. The method is easy to perform as a one-step-procedure without requiring any *in vitro* cell culturing. The shortcoming of the study was that the urothelium was only loosely attached.

6.2 STUDY II

- Smooth muscle cells from minced detrusor proliferate in the same way as transplanted minced urothelium when transplanted around a mould. However, co-transplantation of minced detrusor and urothelium at the same time does not create a viable condition for the urotehlial cells. Hence, this method could not be used to construct a more stable prototyp for creating a catheterizable bladder conduit.

6.3 STUDY III

- The sex ratio was equal and the prevalence stable over time. Birth descriptive data were comparabel with those of controls and BE was usually an isolated congenital malformation. Congenital inguinal hernia and undescended testis were characteristics of the BE patients to a higher degree than in the general population.
  The only potentially associated maternal risk factor was advanced age.

6.4 STUDY IV

- The incidence of bladder cancer and psychiatric disorders was the same as in the general Swedish population. Mortality was low and sensory neurogenic hearing disorders indicated a possible genetic association in a few cases, based on previous genetic studies. No differences concerning partnership could be established, but significantly fewer cases conceived biological children. The overall educational level was high.

7 FUTURE ASPECTS

Despite the developments in tissue engineering technologies and regenerative medicine, current clinical applications in the field of bladder augmentation or autologous bladder conduits are rare. Many research challenges still seem to remain unsolved. The main obstacle is the significant risk of serious complications. This is partly due to the risk of graft ischemia and failure of tissue contraction since there are no available scaffolds to date with properties that enhance vessel or nerve ingrowth. Tissue engineering techniques and regenerative medicine have also proved to be both more complex and more expensive than previously imagined thus reducing a widespread adoption of the technique.

Regenerative medicine has the potential to be the future in many fields of medicine. There are still many obstacles to encounter and many questions to be answered, but in time, we will get there. The answer lies within our own bodies. We just have to learn how to interpret the signals.

With the advances in pediatric medicine and reconstructive surgery, the morbidity and mortality associated with BE have decreased drastically. In the Third World, however, one may come across these patients at an older age, because there is often a delay before they seek medical advice. We still need to establish and to apply national surveillance programs that run through the whole life span of a patient with BE, including follow-up for bladder cancer with perhaps yearly cystoscopy. We also need to establish a clear definition of continence, and to establish good follow-up for psychosexual function and fertility issues. Surgical methods also have to be refined to minimize further harm to delicate tissues.

I personally do not believe in an enhancement in prenatal detection for the purpose of terminating BE fetuses. I believe that this method is a way of hiding medical and surgical imperfection in an otherwise healthy human being with resources and potentials, as valuable as those of any other human being. In contrast we have to elucidate problematic issues with BE, and strive to solve surgical and medical problems. BE is a perfect chance and opportunity to learn more about the human body and to utilize successful surgical techniques for use in otherwise healthy people.

For the future and the best outcome for BE patients, we have to continue our work with surgical improvements and clinical follow-ups for enhanced success rates regarding incontinence, augmentation without risk of cancer, improvements in the cosmetic and

functional appearance of genitalia, as well as psychosocial support. Considering the rarity of the malformation, this is best done on a national or multinational basis with standardized treatment programs and surgery at highly specialized institutions in childhood, adolescence and adult life.

We have to add on and combine the benefits of new technological developments, such as regenerative medicine and there must be a close relationship between biotechnology and the patient.

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