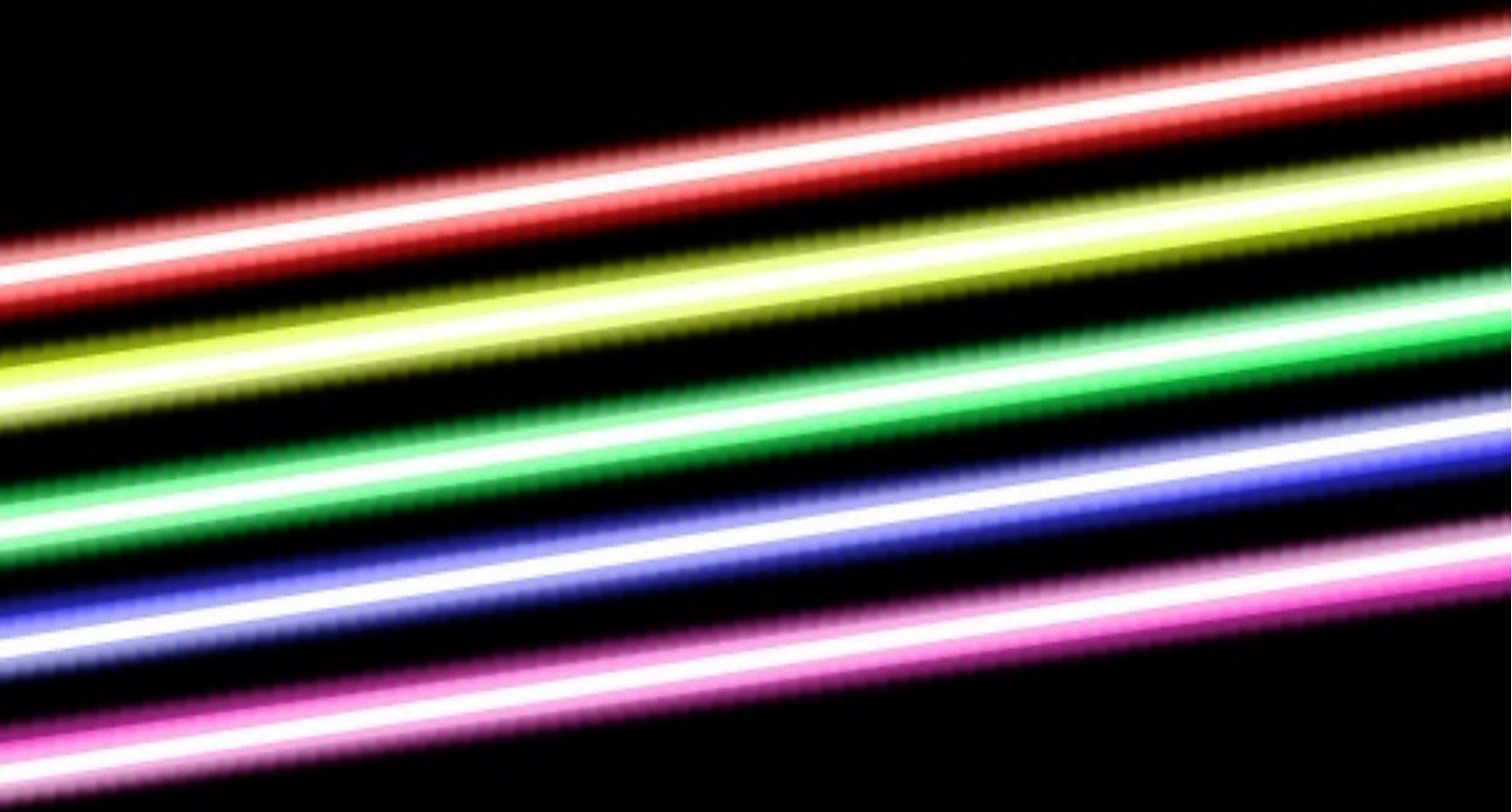
Novel treatment modalities for pelvic floor dysfunction

Katerina Mackova



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Charles University in Prague, Czechia
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NOVEL TREATMENT MODALITIES FOR PELVIC FLOOR DYSFUNCTION

Katerina MACKOVA

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The picture on the cover represents LASERS. This picture was made by K. Mackova.

For Charles University in Prague:

Prohlašuji, že jsem závěrečnou práci zpracovávala samostatně a že jsem řádně uvedla a citovala všechny použité prameny a literatuře. Současně prohlašuji, že práce nebyla využita k získání jiného nebo stejného titulu. Souhlasím s trvalým uložením elektronické verze mé práce v databázi systému meziuniverzitního projektu Theses.cz za účelem soustavné kontroly podobnosti kvalifikačních prací.

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Katerina Mackova

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LIST OF ABBREVIATION:

ø - Diameter
AE - Adverse Events
ACM - Acellular Collagen Matrix
APFQ - Australian Pelvic Floor Questionnaire
ARRIVE - Animal Research: Reporting of In Vivo Experiments
A-SMA - α- Smooth Muscle Actin
bFGF - Basic Fibroblast Growth Factor
BMI - Body Mass Index
CD - Cluster of Differentiation
cDNA - complementary Deoxyribonucleic Acid
CI - Confidence Interval
CO2 - Carbon Dioxide
COS - Core Outcome Set
d - day
Er: YAG - Erbium Yttrium Aluminium Garnet
ECM - Extracellular Matrix
eMSC - endometrial Mesenchymal Stem/stromal Cells
ET - Epithelial Thickness
EU - European Union
FBGCs - Foreign Body Giant Cells
FDA - U.S. Food and Drug Administration
FIBEr - Flanders Institute for Biomechanical Experimentation
Foxp3 - Forkhead box P3
FSFI - The Female Sexual Function Index
GAGs - glycosamin glycans
GRC - Graft Related Complications
GSM - Genitourinary Syndrome of Menopause
h - hours
hASCs – human Adipose-derived Stem Cells
H&E - Hematoxylin & Eosin
HRT - Hormone Replacement Therapy
IC - Iliocaudalis
i.e. - id est
ICIQ-UI SF - International Consultation on Incontinence Questionnaire-Urinary Incontinence Short Form
ICS - International Continence Society
IGF - Insulin Growth Factor
IHC - Immunohistochemistry
IL - Interleukin
IQR - Interquartile Range
iNOS - inducible Nitric Oxide Synthase
ISI - incontinence symptoms scores
IUGA - International Urogynecological Association
IUC - International Urogynaecological Association's Consultation
IV- Intravenous
Kg - Kilogram
L - Liter
LA - Levator Ani
LAM - Levator Ani Muscle
M1 - Macrophage type 1
M2 - Macrophage type 2
MeSH - Medical Subject Headings
mL - millilitre
mm - millimetre
MMP - Matrix Metalloproteinase
MRI - Magnetic Resonance Imaging
mRNA - messenger Ribonucleic Acid
NHP - Nonhuman Primates
N - Newton
NTR - Native Tissue Repair
OAB - Overactive Bladder
OABSS - Overactive Bladder Symptom Score
OCT - Optical Coherence Tomography
OVX - Ovariectomy
PAF - Paraffin
PAS - Periodic Acid Schiff
PBS - Phosphate Buffered Saline
PCR - Polymerase Chain Reaction
PFD - Pelvic Floor Disorders
PFM - Pelvic Floor Muscles
PFMT - Pelvic Floor Muscle Therapy
PISQ12 - Pelvic Organ Prolapse/Urinary Incontinence Sexual Questionnaire 12
PLA - Poly-L-lactic Acid
PNC - Pudendal Nerve Crush
POP - Pelvic Organ Prolapse
POPDI6 - Pelvic Organ Prolapse Distress Inventory-6
POP-Q - Pelvic Organ Prolapse Quantification system
PP - Polypropylene
PRISMA - Preferred Reporting Items for Systematic reviews and Meta-analyses
MUS - midurthral sling
PRP - Platelet-rich Plasma
PROSPERO - International Prospective Register of Systematic Reviews
PVDF - Polyvinylidene Fluoride
RCT - Randomized Controlled Trial
RNA - Ribonucleic Acid
ROBINS - Risk of Bias in Non-randomised Studies of Interventions
RR - Relative Risk
SD - Standard Deviation
SEM - Scanning Electron Microscope
SIS - Small Intestinal Submucosa
SUI - Stress Urinary Incontinence
SVD - Simulated Vaginal Delivery
TGF - Tumor Growth Factor
TNF - Tumor Necrosis Factor
TOT - Transobturator Tape
TVT - Tension Free Vaginal Tape
UI - Urinary Incontinence
UTI - Urinary Tract Infection
µm - micrometre
VAS - Pain Visual Analog scale
VD - Vaginal Distension

CHAPTER 1

Introduction and study aims

CHAPTER 1 - INTRODUCTION AND STUDY AIMS

1.1. Aging and age-related disease

Thanks to better nutrition and progress in medicine in developed countries during the last 100 years, life expectancy has raised rapidly (Fig. 1). In Belgium, for example, it raised from 49.8 years in 1919 to 81.6 years in 2019, which is an increase of 64% or in absolute number of 31.8 years[1]. This increase is not always paralleled with sustained functioning. Indeed, there are many age-related diseases decreasing quality of life in the aging population, including cardiovascular diseases, osteoporosis, or arthrosis, as well as conditions that affect only females. Of relevance to this thesis are genitourinary syndrome of the menopause (GSM) and pelvic floor disorders (PFD).

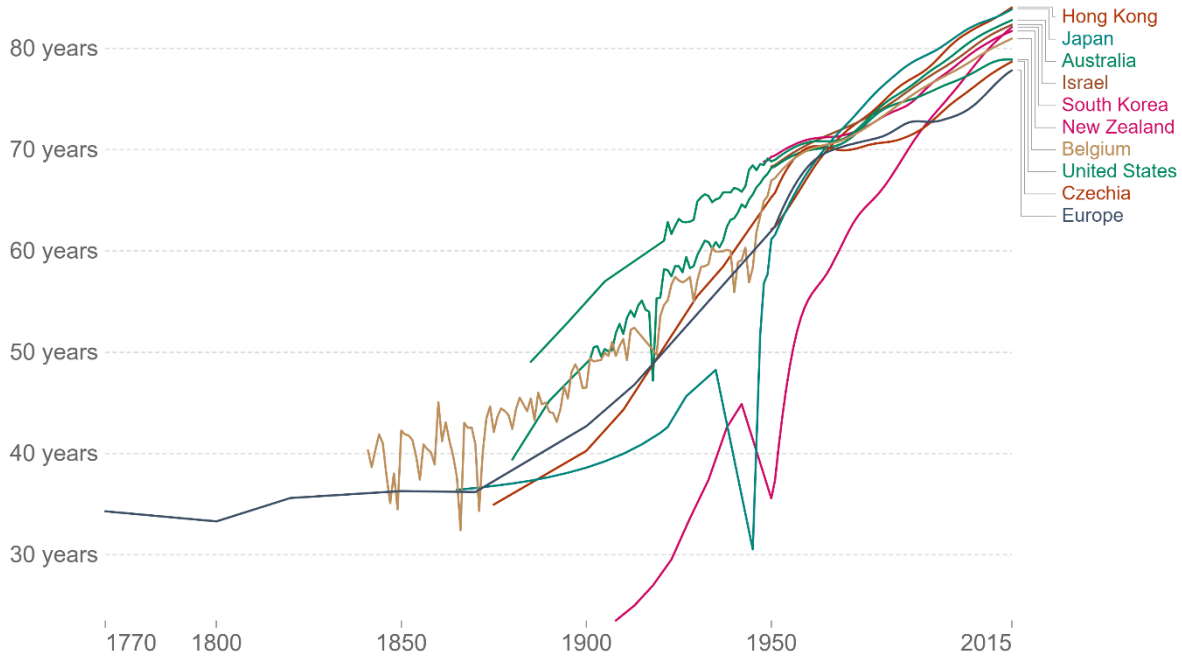

Figure 1: Life expectancy in selected developed countries. Adapted from Our World In Data[1] Riley (2005), Clio Infra (2015), and UN Population Division (2019). Note: Shown is period life expectancy at birth, the average number of years a new-born would live if the pattern of mortality in the given year were to stay the same throughout its life.

1.2. Pelvic floor disorders

PFD comprise three often interrelated conditions: pelvic organ prolapse (POP), urinary incontinence (UI) and anal incontinence (AI). The underlying pathophysiology is degeneration of the supportive structures of the pelvic floor and their function. The inciting factors, as we understand today, are pregnancy and delivery, aging, next to a whole series of lifestyle factors (Fig. 2).[2, 3]

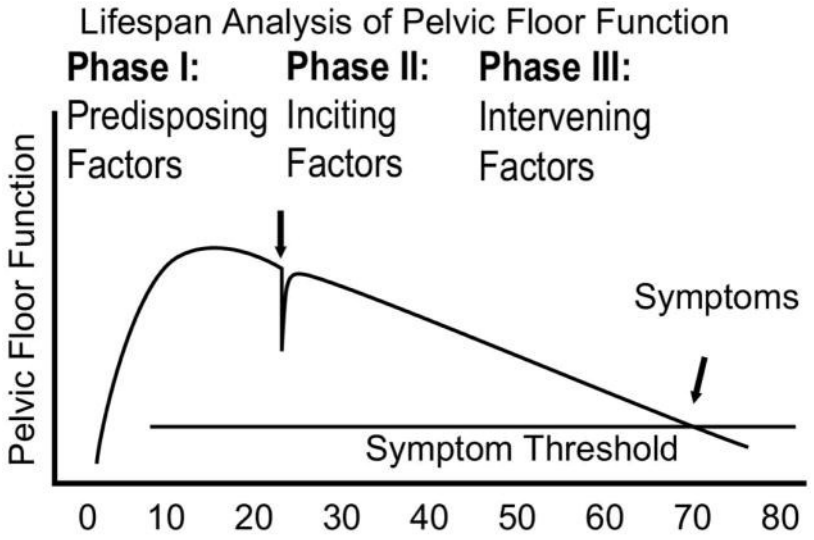

Figure 2: Life span model causal factors of pelvic floor disorders. (DeLancey et al, 2008)[3] Reproduced with permission of the publisher.

1.2.1 Pelvic organ prolapse

Pelvic organ prolapse (POP) is a highly prevalent disorder in the general female population with a prevalence, based on clinical examination, exceeding 30%. The prevalence based on the symptom of a vaginal bulge, ranges between 5 and 10%[4]. The diagnosis of Pelvic Organ Prolapse (POP) involves the identification of descent of one or more of the following (Figure 3): the anterior vaginal wall (central, paravaginal or a combination), posterior vaginal wall (rectocele), the uterus (cervix) or, in its absence, the apex of the vagina (vaginal vault or cuff). The presence of any such sign may correlate with relevant POP symptoms. The feeling of heaviness or a lump in the genital area is the most commonly reported symptom, but urinary incontinence, constipation, faecal incontinence and several other complaints are reported by patients with POP as well[5]. Symptoms are generally worse after long periods of standing or exercise and better when gravity does not interfere anymore, e.g. lying supine. Prolapse may also be more prominent during abdominal straining, e.g. during defecation or when lifting heavy weights[6]. POP is caused by a combinations of factors, including pregnancy, childbirth, connective tissue disorders, pelvic neuropathies, congenital factors, pelvic surgery, and miscellaneous factors such as obesity, respiratory disorders, occupational and recreational stress, and hypoestrogenism[7]. Incidence and costs related to it, are growing in the developed world with an aging population and longer life expectancy[8].

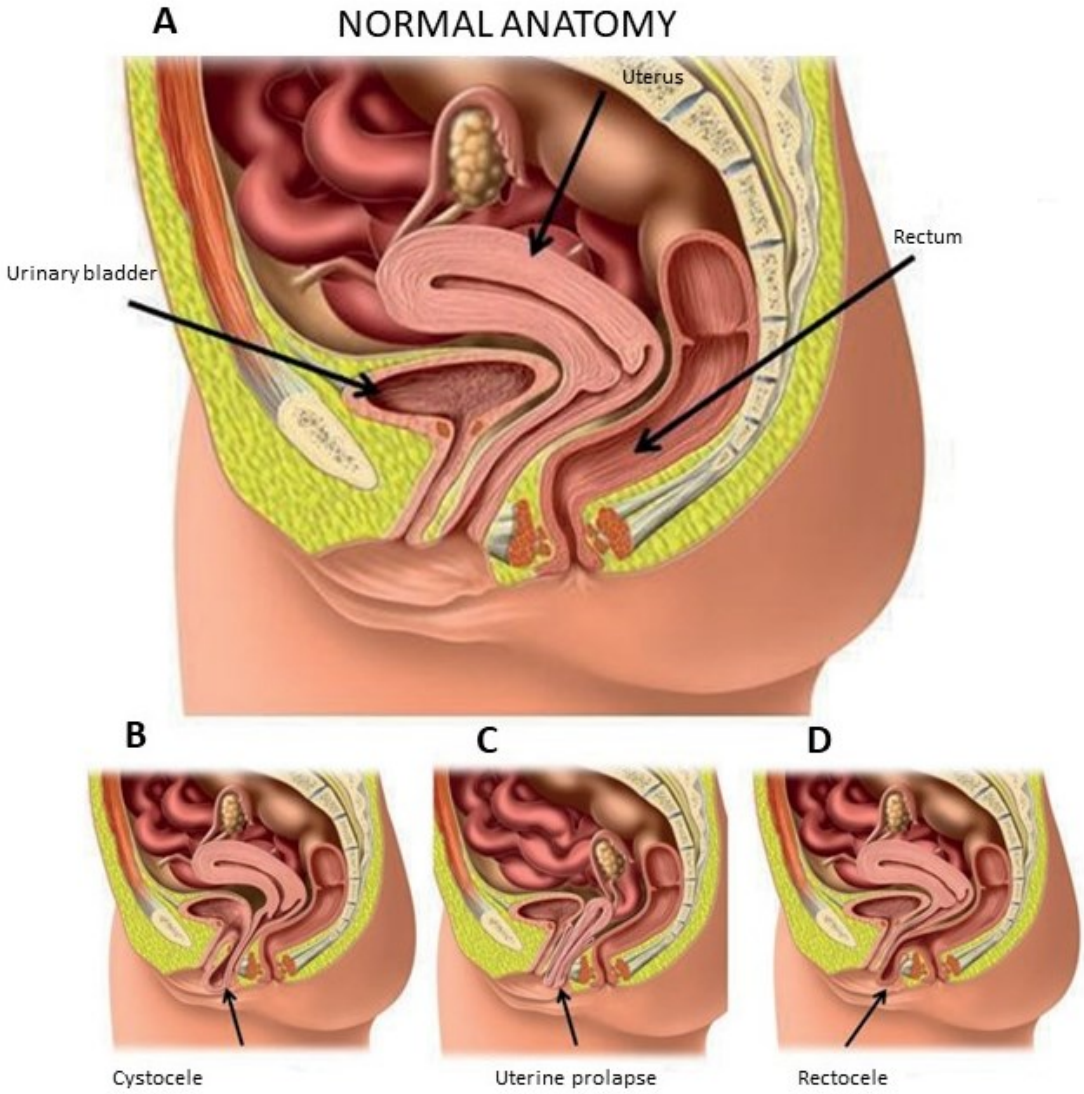

Figure 3: Schematic drawing showing (A) normal pelvic floor anatomy, (B) anterior, (C) middle compartment and (D) posterior prolapse. Illustration - DreamTeam.

1.2.2 Urinary incontinence

Urinary incontinence (UI) is defined as the complaint of involuntary loss of urine (symptom) or the observations of involuntary loss of urine on examination[9]. The range of prevalence reported for urinary incontinence of any subtype in adult women is broad (5–72%), with studies converging on a prevalence of approximately 30%[10]. The three most common types of urinary incontinence are Stress Urinary Incontinence (SUI), Urge Urinary Incontinence (UUI) and Mixed Urinary Incontinence (MUI). SUI is a condition of involuntary loss of urine on effort or physical exertion including sporting activities, or on sneezing or coughing. UUI is a condition where the involuntary loss of urine is associated with a feeling of urgency. Urgency is characterised by the sensation of a sudden, compelling desire to void which is difficult to defer. When both UUI and SI are present, the term mixed urinary incontinence (MUI) is used[11].

1.2.3 Anal incontinence

Faecal Incontinence (FI) is the involuntary loss of faeces – solid or liquid. Anal Incontinence (AI) includes these events as well as the involuntary loss of flatus, which is perceived by many patients to be an equally disabling disorder[4]. The prevalence of AI ranges between 5 and 15% depending on what exact symptoms are taken into

account[4]. Injury to the pudendal nerve or sphincter muscle from prior obstetric trauma is described as the primary risk factor in the female population. Treatment of AI starts with medical therapy and physical therapy but may also involve surgical management[12]. In this work, AI was not studied, hence only included in this introduction for completeness. However, novel treatment modalities as LASER are also being suggested in this field and will be interesting to study in the future[13].

1.2.4 Therapy of PFD

The treatment of PFD may be either conservative - pelvic muscle training, behavioural training, pessaries for POP, and pharmacotherapy for OAB, or surgical. Each of the treatment modalities has its specific benefits and possible adverse events (AE). Both pelvic floor muscle training (PFM) and behavioural training (bladder training, fluid management and others) are safe and cheap options. They also have a low risk of adverse events. Eventual harm could be caused by incorrect training, which may lead to worsening of symptoms or new pain occurrence[14]. For POP, pessaries are another non-surgical option. These are also having risks of AE, including bleeding, extrusion, disturbing vaginal discharge, pain and constipation. AE occur quite often, up to 60% during long term use, and may eventually lead to discontinuation of pessary use[15, 16]. Antimuscarinic (anticholinergic) drugs and mirabegron (the first clinically available beta-3 agonist; in some countries, other beta 3 agonists are also available) are the most frequently used drugs for UUI. For anticholinergics, dry mouth is the commonest side effect, though constipation, blurred vision, fatigue, and cognitive dysfunction may occur. Mirabegron, on the other hand, may increase blood pressure, therefore is contraindicated in patients with severe uncontrolled hypertension[17].

Besides conservative therapies, surgery is also an option for UI and POP. Surgery may be performed with insertion of implants or without, i.e. using the patient's own tissue and sutures. Any surgery may cause AE, more frequently when mesh is used[18, 19].

There are also AE that are uniquely associated to mesh use, such as exposure and extrusion[20]. Other possible AE may be tied to other factors as well, including pain (in the abdomen, pelvis, groins, vulva or vagina, and lower limbs), dyspareunia, infection, voiding dysfunction, and other functional bladder and bowel symptoms. Additionally, psychological sequelae as a result of physical problems are common too[21]. The increasing number of reports of AE following vaginal insertion of implants has led to the withdrawal or restricted use of vaginal mesh, and in some countries of MUS (midurthral sling) as well [22, 23]. Lastly, mesh enhanced surgery may also fail, so that symptoms can reoccur. Because all this, novel treatment modalities, such as LASER therapy, or the use of novel implants based on alternative polymers and/or textile structures should be considered, which one can, and, according to us, should test preclinically (Table 1)[24].

Table 1: Anticipated timeline of the current proposal for the introduction of novel devices into the market. Adapted from Slack et al. (2012)[24]

Steps	Goals	Timeline
Pre-marketing, nonclinical		
1. Preclinical file	Accurate description of product—toxicity studies for new polymers	0–6 months
2. Preclinical testing-animal	Host inflammatory response	0–12 months
3. Cadaveric studies	Anatomical documentation	6–12 months
Pre-marketing, clinical		
4. Clinical studies: phase II trial	Efficacy study Long-term safety	12–24 months Ongoing
Post-marketing		
5. Clinical studies: temporary registry "Yellow card"—MAUDE reporting? Recommended: RCT	Surveillance study (*n*=1,000) Self-reporting on a larger scale Should prove whether product/procedure is advantageous/competitive	30–42 months Ongoing Should be conceived as early as possible

1.3. Genitourinary syndrome of menopause

Genitourinary syndrome of menopause (GSM) is a collection of signs and symptoms in the lower urogenital tract associated with low-oestrogen levels[25]. Main subjective complaints are genital burning, itching, irritation, lack of lubrication, sexual discomfort or pain, urinary symptoms of urgency, dysuria and recurrent urinary tract infections[25]. Objectively, low circulating oestrogen levels lead to thinning of the vaginal and uro-epithelium, an increase in vaginal pH, a decrease in collagen and tissue elasticity and fewer blood vessels[26]. Though vaginal atrophy may be present in up-to 90% of menopausal women, only 30% has subjective complaints[27]. GSM may also develop in younger women, e.g. when hormonal sensitive tumours are treated either with surgical castration or by antioestrogen treatment[28].

1.3.1 Therapy of GSM

Mild GSM may be treated with a change in lifestyle[29, 30], lubricants[31] and herbal remedies[32, 33]. For mild to severe GSM, topical or systemic hormonal treatment is effective[34]. However, not all women can or wish to use hormones because of its true or perceived risks, including an increased risk of uterine or breast cancer[35]. Therefore, LASER therapy has been suggested as a novel treatment modality with better risk profile[36, 37].

1.4. Novel treatment modalities

The novel treatment modalities we will investigate, are, for GSM, POP and UI LASER therapy, and for POP and UI, we will test novel textile implants.

1.4.1 LASERS

Recently, non-ablative LASER therapy has been proposed as an alternative non-invasive treatment for GSM, POP and UI, using either erbium-doped yttrium aluminium garnet (Er:YAG) or carbon dioxide (CO_2). Both laser sources were previously used in dermatology, e.g. for treating wrinkles by "skin rejuvenation"[38]. LASER may partially reverse skin atrophy trough activation of heat shock proteins, neoangiogenesis, neocollagenogenesis and increase of epithelial thickness[36, 39-41]. Laser companies are claiming that lasers used vaginally and/or within the urethra may have a similar effect as on the skin, hence are an alternative to surgery and/or pharmacotherapy, offering similar or better effects and with minimal AE[42, 43]. Laser has promptly spread over hospitals and private gynaecological practices, with prices up-to 1000€[44] for a single application, however evidence for its use is lacking. For example of LASER device see Fig. 4.

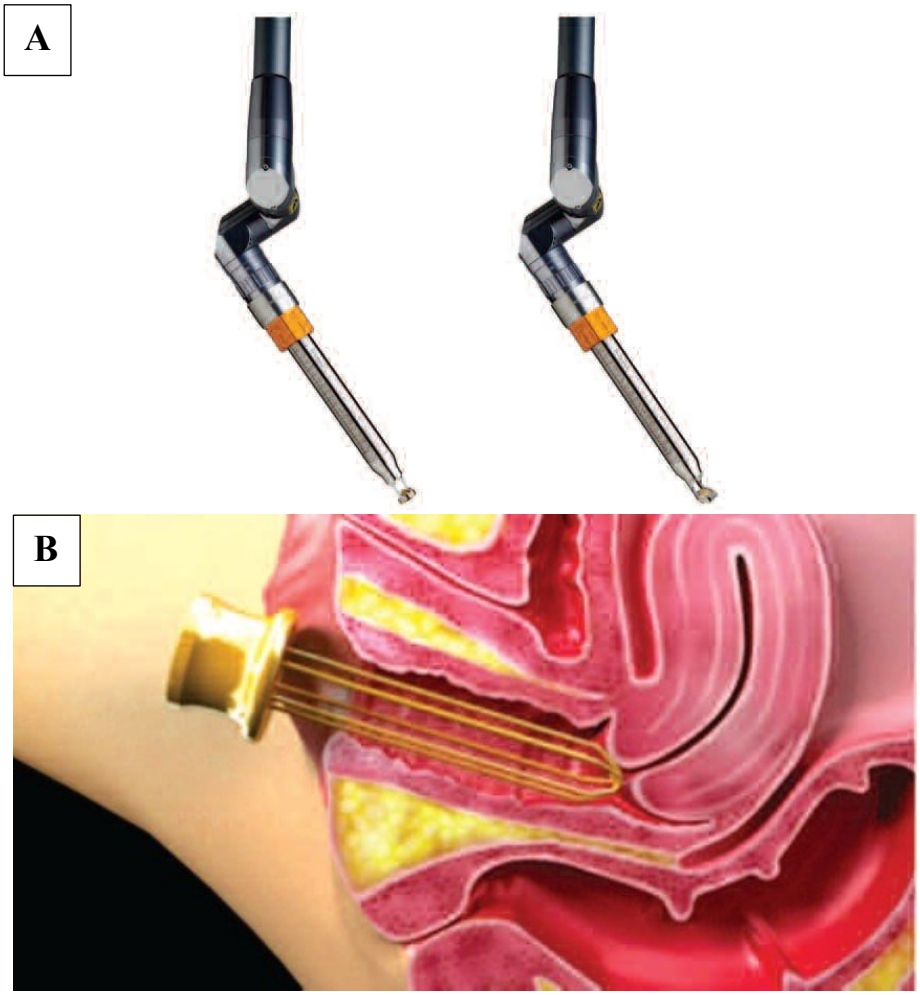

Figure 4: A – example of a vaginal laser probe (360° scanning scope - left and the 90° scanning scope -right). B – example of a vaginal speculum for laser application. Adapted from Lee et al. (2014)[45].
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We performed initial experiments to determine whether the ewe can serve as a menopausal model for vaginal atrophy and whether it is feasible to apply vaginal LASER[46]. That work showed that sheep develop progressive vaginal atrophy after surgical castration, and LASER does not induce long-lasting (90 d) effects on vaginal tissues neither reverse vaginal atrophy[46].

1.4.2 Textile implants

Textile implants, usually referred to as meshes (Fig. 5), have been introduced into the field of reconstructive surgery in the second half of the 20th century[47]. Their purpose was to reduce the high recurrence rates following "native tissue" repair of defects [48, 49]. First used in abdominal and inguinal hernia surgery, mesh was later applied in the treatment of urinary incontinence and pelvic organ prolapse (POP)[50].

Unfortunately, mesh may induce local short-term and long-term AE, including pain or feeling of a foreign body, exposure (often referred to as erosion), extrusion, or infection[51]. Depending on the location of implantation, this may cause functional problems like subfertility, dyspareunia, infection, functional bladder, and bowel symptoms.

Eventually, the use of mesh is dependent on the balance of the reduction in risk for recurrence, and the added risk for implant-related complications. The frequency of reported AE differs widely in literature dependent on a definition of the AE and on the time interval from the surgery. For vaginally inserted mesh for POP repair, the risk for complications seems higher[22, 23, 52] than the ones reported when implants for prolapse are inserted abdominally, e.g. for sacrocolpopexy or rectopexy[53], or mid-urethral slings, used for UI but also vaginally inserted[54].

Nevertheless, considering the high prevalence of POP and UI, and the need for their surgical correction[55-57], there will be always a need for mesh in reconstructive surgery. Its use may grow as the population ages, lives longer and more active lifes[58], and unfortunately also includes a higher number of obese patients, who are at increased risks for the conditions above[59, 60]. In view of that health authorities recommended more research to understand the pathogenesis of mesh complications[61]. Several factors associated with AE have been identified, including patient` characteristics, surgical skills, as well as implant properties, such as mechanical properties, mesh weight, pore size and stability, polymer used as well as the manufacturing process[51]. Based on differences in weight, pore size and polymers used, three different mesh classifications were established (Table 2). The first one by Amid is based on the porosity (Amid, 1997), the second one on the mesh weight (Coda, 2012), and a third more complex classification is based on wider spectrum of recent implants available on the market (Klinge, 2012).

Today, there seems to be a consensus that implants should be macroporous, light weight, stable, and once incorporated into the host, have mechanical properties close to those of native tissue[47, 62]. These factors are closely inter-related but so far, there has been no study that tested the impact of the choice of polymer itself in meshes of otherwise identical structure.

Table 2: Implant materials classification by: A. Amid, 1997 B. Coda, 2012 and C. Klinge, 2012

A/Classification by Amid	Pore size	Component	Product
Type I	Macroporous (>75 microns)	Polypropylene Polypropylene/polyglactin 910 Polyglactin 910	Marlex, Atrium, Prolene, Trelex Gynemesh, Vypro, Vicryl
Type II	Microporous (<75 microns)	Expanded polytetrafluoroethylene (PTFE)	Goretex
Type III	Macroporous with multifilamentous or microporous component	Dacron Polytetrafluoroethylene (PTFE) Polypropylene	Mersilene Teflon Surgipro
Type IV	Submicronic pores/sheets (< 1 micron)	Polypropylene sheet Pericardial membrane	Cellgard Preclude

B/Classification by Coda	Weight	Company product (weight)
Class I: Ultra-light	< 35 g/m^2	Herniamesh, HERMESH 7 (19); Aspide, SURGIMESH XLIGHT (27.5); Textile Hi-Tec, PARP PH (28); Surgical Ioc, PROMESH LIGHT (28); Herniamesh, HERMESH 8 (30); Cousin, BIOMESH P8 (32)
Class II: Light	35 - 69 g/m^2	B. Braun, OPTILENE MESH LP (36); Herniamesh, HERMESH 6 (48); DI.PRO, EVOLUTION (50); TransEasy Medical Tech, PMM (60); Microval, 2D KNITTED LW (64)
Class III: Standard	70 - 139 g/m^2	Abiss, CRISTALENE (70); Herniamesh, HERTRA 9 (88); Bard, BARD MESH (90); Herniamesh, HERMESH 3 (127); Taisier-Med, EGYMESH 2 (127); Serag Wiessner, SERAMESH SO (130);
Class IV: Heavy	≥ 140 g/m^2	FiraMedicale, FIRAMESH SEMI-RIGID (175); Herniamesh, HERTRA 2 (177); Gis, RM2 (180) Gis, RM3 (220); FiraMedicale, FIRAMESH RIGID (220); Herniamesh, HERTRA 0 (242); Gis, RM3 (250)

C/Classification by Klinge		Sub-division or characterization	Example of mesh
Class I:	Large pore meshes (characterized by a textile porosity of >60% or an effective porosity of >0%)	Ia) Monofilament Ib) Multifilament Ic) Mixed structure or polymer (e.g. absorbable + non- absorbable, or different non-absorbable)	Vypro Ultrapro Ti-mesh Mersilene
Class II:	Small pore meshes (characterized by a textile porosity of <60% and without any effective porosity)	Ia) Monofilament IIb) Multifilament IIc) Mixed structure or polymer.	Marlex Prolene Atrium Surgipro
Class III:	Meshes with special features	Porous meshes with special features, e.g. to prevent adhesions	Sepramesh
Class IV:	Meshes with films	Film-like meshes without porosity, submicronic pore size or secondarily excised pores	ePTFE
Class V:	3D meshes	Pre-shaped, pre-formed, or 3D devices	Electrospun (PCL,PU)
Class VI:	Biologicals	VIa) Non-cross-linked VIb) Cross-linked VIc) Special features	Surgisis

All implant materials above can be absorbable or non-absorbable

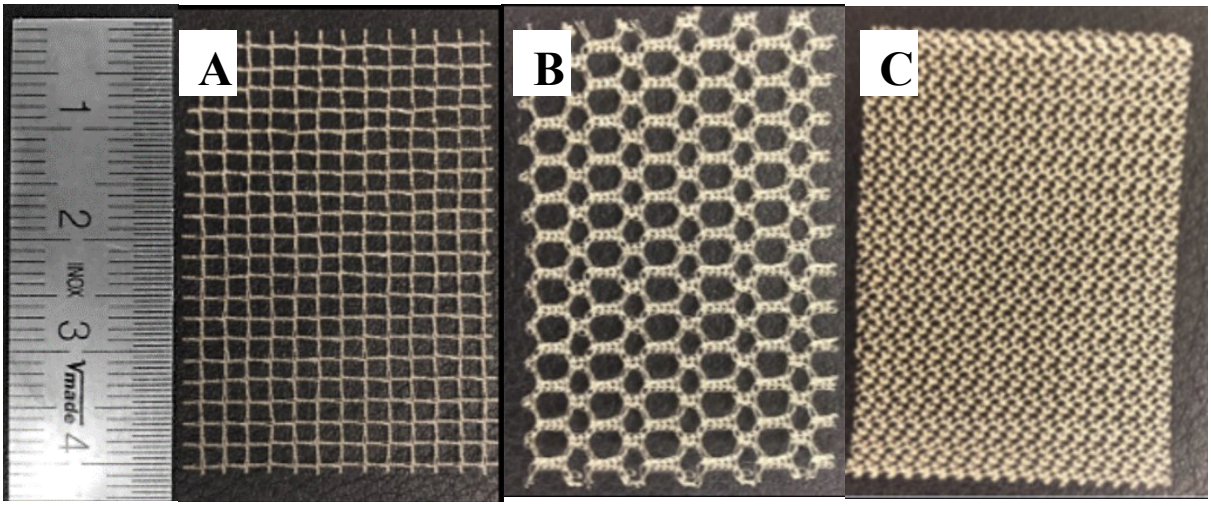

Figure 5: Macroscopical figures of 3 different textile implants. A. Restorelle, B. Dynamesh-CICAT and C. Marlex.

1.4.3 Animal models

For testing some novel treatment modalities in a preclinical environment, a wide spectrum of animal models can be used. In Chapter 2 and 3 we will go into detail on the relevance of animal models, and their use in research on implants, and the effect of (iatrogenic) menopause.

1.5. Summary of objectives

The overall aim of my research was to experimentally assess novel treatment modalities for PFD and GSM. The research started with an extensive literature search, prior to the specific animal experiments.

The first aim was to summarize current knowledge on animal models in the study of in the pathogenesis and treatment of selected PFD (**Chapter 2 and 3**), and on the use of LASER therapy for GSM (**Chapter 4**) and PFD (**Chapter 5**).

The second aim was to study preclinically the effects of vaginal LASER therapy, using the sheep menopausal model (**Chapter 6**).

The third aim was to preclinically test a novel mesh material for pelvic floor surgery, i.e. a PVDF mesh, which is implanted in the rat abdominal hernia model and compare it to standard polypropylene mesh (**Chapter 7**). In this experiment, the textile structure of both implants was kept identical, so that ultimately the only variable was the polymer.

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Chapter 2

Animal Models for Pelvic Organ Prolapse: Systematic Review

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ABSTRACT

Introduction and hypothesis: We aimed to summarize the knowledge on the pathogenesis of pelvic organ prolapse (POP) generated in animal models.

Methods: We searched MEDLINE, Embase, Cochrane and the Web of Science to establish what animal models are used in the study of suggested risk factors for the development of POP, including pregnancy, labour, delivery, parity, aging and menopause. Lack of methodological uniformity precluded meta-analysis hence results are presented as a narrative review.

Results: 7,426 studies were identified of which 51 were included in the analysis. Pregnancy has a measurable and consistent effect across species. In rats, simulated vaginal delivery induces structural changes in the pelvic floor, without complete recovery of the vaginal muscular layer and its microvasculature, though it does not induce POP. In sheep, first vaginal delivery has a measurable effect on vaginal compliance; measured effects of additional deliveries are inconsistent. Squirrel monkeys can develop POP. Denervation of their levator ani muscle facilitates this process in animals that delivered vaginally. The models used do not develop spontaneous menopause, so it is induced by ovariectomy. Effects of menopause depend on the age at ovariectomy and the interval to measurement. In several species menopause is associated with an increase in collagen content on the longer term. In rodents there were no measurable effects of age apart of elastin changes. We found no usable data for other species.

Conclusion: In several species there are measurable effects of pregnancy, delivery and iatrogenic menopause. Squirrel monkeys can develop spontaneous prolapse.

Key Words: ovariectomy, pregnancy, parity, pathophysiology, age, menopause

Introduction

Pelvic Organ Prolapse (POP) is the abnormal downward descent of pelvic organs, i.e., the bladder, uterus and/or the rectum, resulting in a protrusion through the vagina [63]. POP is quite common, even though many women are asymptomatic [64]. POP may be associated with a wide range of symptoms, such as the sensation of vaginal bulging, urinary and more rarely also fecal incontinence or evacuation problems, pain, and dyspareunia. Patients with significant prolapse also have a significantly reduction in their quality of life [65].

Several risk factors for the later occurrence of POP have been named. The most important ones are parity, pregnancy, obesity and aging [63]. Its long-time course and the complex and multifaceted nature of this disorder make it difficult to study the condition clinically. As part of the International Urogynaecological Association's Consultation (IUC) initiative, the Committee drafting a report on the pathophysiology of POP, decided to review the literature on animal models with that perspective. Animal models are convenient as they allow for complex experimental design or discounting an abundance of interfering co-factors as in the clinical situation. Ideally, in these models the life events considered as risk factors in women, should result in comparable structural and functional changes in the pelvic floor. Finding an optimal model is challenging, since humans are bipedal, have no tail, and in the context of pregnancy and delivery as a risk factor, the fetal head is relatively large compared to the pelvic dimensions, making vaginal delivery more traumatic compared to other species. Conversely, nearly all animals are quadrupeds, with a different pelvic floor musculature including a functional tail, and they have a different birth process [66].

There are occasional reports of naturally occurring vaginal prolapse in different animal species, including rabbits [67], sheep [68, 69], a number of nonhuman primates (NHP) [70-72], cows [73, 74], pigs [75], dogs [76, 77], cats [78] and buffalos [79-81]. Most of the research in larger animal models has been done in sheep and squirrel monkeys, and more detail will be provided on findings in these species. This review will also list the work done in smaller species, but we do not cover genetic models. We first introduce clinicians to generic information on the species used in translational research on the pathophysiology of POP for further guidance.

Reproduction cycle and comparative pelvic anatomy of species used in the study of risk factors for POP

The complex supportive system of the pelvic floor is assumed to be crucial to cope with the forces exerted when bipeds are standing upright. In quadrupeds levator ani (LA) is responsible for tail movements [82]. In those animals the bulk of the body weight is oriented perpendicular to the spine. As a consequence, the main support for pelvic organs is bony in nature and formed by the pubic bones and ischia. Evolution to bipedalism shifted the load of the body weight parallel to the spine and the spine, pelvis, and hips are thought to be adapting accordingly. As a result, the pelvic floor became horizontal and critical for continence and the prevention of POP. Compared to quadrupeds, humans have a more complex pelvic floor and LA muscle complex [83].

Rodents:

The small size of rodents, the difference in posture, and the small size of the fetus, make prolapse unlikely and renders the rodent model not very appropriate for studying conditions that predispose one to developing prolapse. However, their ease of handling, short lifespan and relatively low cost, with less ethical constraints than higher species, are advantages [84].

Rodents have a predictable and short estrous cycle (mice: 2-5d; rats: 4d) and length of gestation (mice:19-21d; rats:21-23d) that make POP development less time intensive to study [85].

Anatomically, the gross connective tissue anatomy of the rodent pelvis is similar to that of humans[86]. The rodent pelvis has uterosacral ligaments that also attach the upper vagina to the lower spine. Paravaginal attachments insert on a dense band of connective tissue extending from the pubic symphysis to the lateral bony pelvis that

serves a role similar to that of the arcus tendineus fascia pelvis in humans. The similarity between the structure and function of the vaginal connective tissues in mice/rats and humans makes them a preferred model when evaluating connective tissue support. Although the LA (referred to as pubocaudalis and iliocaudalis muscles in the rat) is present in rodents, their primary function in rodents seems to be to support the tail, while the connective tissue attachments serve as the vaginal support [86]. One study compared the macro- and micro-anatomy of the round, uterosacral and cardinal ligaments of mice and rats, and they concluded that the rat pelvic floor structures are histologically more comparable to humans than mice [87].

Lagomorphs

The anatomy of the rabbit vagina differs significantly from that of humans. The vagina is relatively long and consists of both an internal and external portion. The upper portion directly communicates with the uterus, has no adjacent connective tissue (unlike the cervix), is histologically more similar to the small intestine than to the vagina, and a large portion of the anterior wall of the external vagina includes the clitoris [84]. Rabbits do not have an oestrus cycle with spontaneous ovulation but require induction of ovulation via vaginal stimulation by coitus. Their gestation period is approximately 31–35 days.

One study compared the *microscopic* and *functional* anatomy of the pelvic floor muscles of the mouse, rat and rabbit using the architectural difference index as an indirect indicator of muscle force generating and moving capacity [88]. It was concluded that pelvic floor muscles of rats were the most similar to humans, followed by those of mice and rabbit.

Sheep

Sheep are suggested as a large-animal alternative to NHP [89]. They are not that expensive, available in large numbers, and they are often used in reproductive medicine studies [84]. Their oestrus cycle is 17 days, which is more similar to that of humans than rabbits, and their average gestation lasts 147 days. Ewes may have prolonged labors with relatively large fetuses, and frequent dystocia [90]. Ewes may have antepartum cervico-vaginal prolapse (1% to 15% in specific flocks) [69, 91]. Its etiology is not well described. Several authors describe signs of milder forms of mid- and lower-vaginal descent *following* pregnancy and delivery [89, 92-99]

The dimensions of the ovine and human vagina are similar in both length and diameter [100]. Additionally, the ovine pelvic architecture relies on three levels of support, similar to those detailed by DeLancey in women [100, 101]. Sheep also have a LA complex and coccygeus muscles but have a different shape and orientation of the pelvis and they lack sacrospinous ligaments and internal obturator muscles. On histology, the ovine vagina has four layers that are similar to those in the human vagina, and a nearly comparable estrogen receptor distribution [100].

Non-human primates (NHP)

NHP have histological, hormonal and anatomical similarities to humans [102, 103]. The reproductive cycle, process of gestation/parturition, large head to pelvic outlet ratio [104] and hormonal effects on the pelvic organs resemble those of humans [84]. NHP also have LA muscles consisting of iliocaudalis (IC), pubocaudalis, and puborectalis muscles, which have analogous functions to the iliococcygeus, puboccygeus, and puborectalis muscles in humans. NHP can develop vaginal prolapse [102, 105]. Disadvantages as an animal model include the long pregnancy and time it takes to develop spontaneous POP, the cost of maintenance, the level of expertise needed to handle them and obviously ethical constraints. We identified studies involving rhesus macaque, squirrel monkeys and baboons. Squirrel monkeys are best studied. Pierce et al. showed that female squirrel monkeys have similar intrapelvic skeletal muscular anatomy to humans and that the LA nerve originates from the S2 spinal root, yet without innervation from the pudendal nerve [103], similar to humans [106]. Their gestation is 153 days, they have disproportionately large fetuses compared to the maternal pelvic outlet (newborn pups have a weight that is 17% of that of their mothers, compared to 8-10% in other primates [107]). Also, labor lasts long (~12 h). Moreover,

when sitting their pelvis is above the ground hence not supported and increasing the strain on it [108]. The frequent stress applied to the pelvic floor may put them at higher risk for developing prolapse than humans [84] .

Material and Methods

Protocol and registration

This review was structured based on the guidance provided in the Preferred Reporting Items for Systematic reviews and Meta-analyses (PRISMA) statement. The research question was: "What animal models for POP are available, and what have they learnt about the relationship between aging, menopause, labour and delivery and POP?"

Information sources, search strategy

A complete computerized literature search was conducted using MEDLINE (PubMed), Embase and the Web of Science including all studies without date and language restriction up to 15^{h} March 2020. The electronic search strategy included both Medical Subject Headings (MeSH) and keywords (Appendix 1). Endnote X8.2 (Clarivate Analytics, Philadelphia, Pennsylvania, United States), Rayyan QCRI and eventually a manual search was used to eliminate duplicate reports. Duplicates were divided into type-I (duplicates among different databases) and type-II (duplicate publications in different journals/issues) duplicates. Reference lists of original articles and topic-related reviews were checked manually to identify further relevant articles.

Eligibility (Studies selection, inclusion and exclusion criteria)

Two authors (MGMCMC and LH) independently screened the abstract, title, or both, of every record retrieved, to determine which study should be assessed further. This was conducted using the Rayyan technology platform, Rayyan QCRI. Any discrepancies were solved through consensus. Eligible studies were those in any experimental animal in which POP was studied, either naturally occurring or provoked. Studies reporting the effects on the vagina, its support apparatus and the LA complex (or its equivalent) were included. Only articles published in English were considered. Studies reporting only qualitative outcomes, genetic models or without a proper control were not included. Review articles, case reports, commentaries, letters to the editor and unpublished articles (i.e. conference abstracts) were excluded. Articles published before than 1999 were also excluded.

Screening Methods and Data Extraction

All potentially relevant articles were assessed as full-text and checked for agreement. The information from these studies was tabulated according to the model, affected tissue, outcome measures and the results.

Given the heterogeneity of the included study designs and outcome measures it was not possible to conduct a metanalysis. In place of this, all studies were appraised for reporting as a narrative review.

SYRCLE's risk of bias tool[109] was used to assess the risk of bias in the included studies. This tool, based on the Cochrane Collaboration RoB Tool[110], aims to assess methodological quality and has been adapted to aspects of bias that play a role in animal experiments. SYRCLE's risk of bias tool consists of a domain-based instrument with 10 items related to 6 types of bias: selection bias, performance bias, detection bias, attrition bias, reporting bias and other biases. These 10 items are organized in subitems in the form of questions that support a "Yes," "No," "Unclear answer." "Yes" refers to low bias with low risk; "No" refers to high bias with high risk; "unclear" is the degree of risk is uncertain. Assessments was done by two independent reviewers, and disagreements was resolved through consensus-oriented discussion or by consulting a third person.

RESULTS

7,421 studies were identified through the search strategy and 5 were identified through the references. After removal of duplicates, 6,363 studies were screened by title and abstract. Of these 6,235 were excluded, as they failed to meet the inclusion criteria. Of the 128 articles assessed for eligibility, 77 were excluded because no full text was available (n=52), there was no assessment of the vagina or pelvic floor muscles (PFM) done (n=8), lack of relevant controls for the risk factor under study (n=8), the studies involved only comparative anatomy (n=3), the study involved male animals (n=1), or not an *in vivo* model (n=5). Eventually, 51 studies were included, and their content summarized in here (Figure 1). Bias assessment was done according to the SYRCLE`s tool. Results are displayed in table 1 for each individual study. Table 2 summarizes the findings for the different models, in terms of passive biomechanics, active contractility testing, morphologic and biochemical changes.

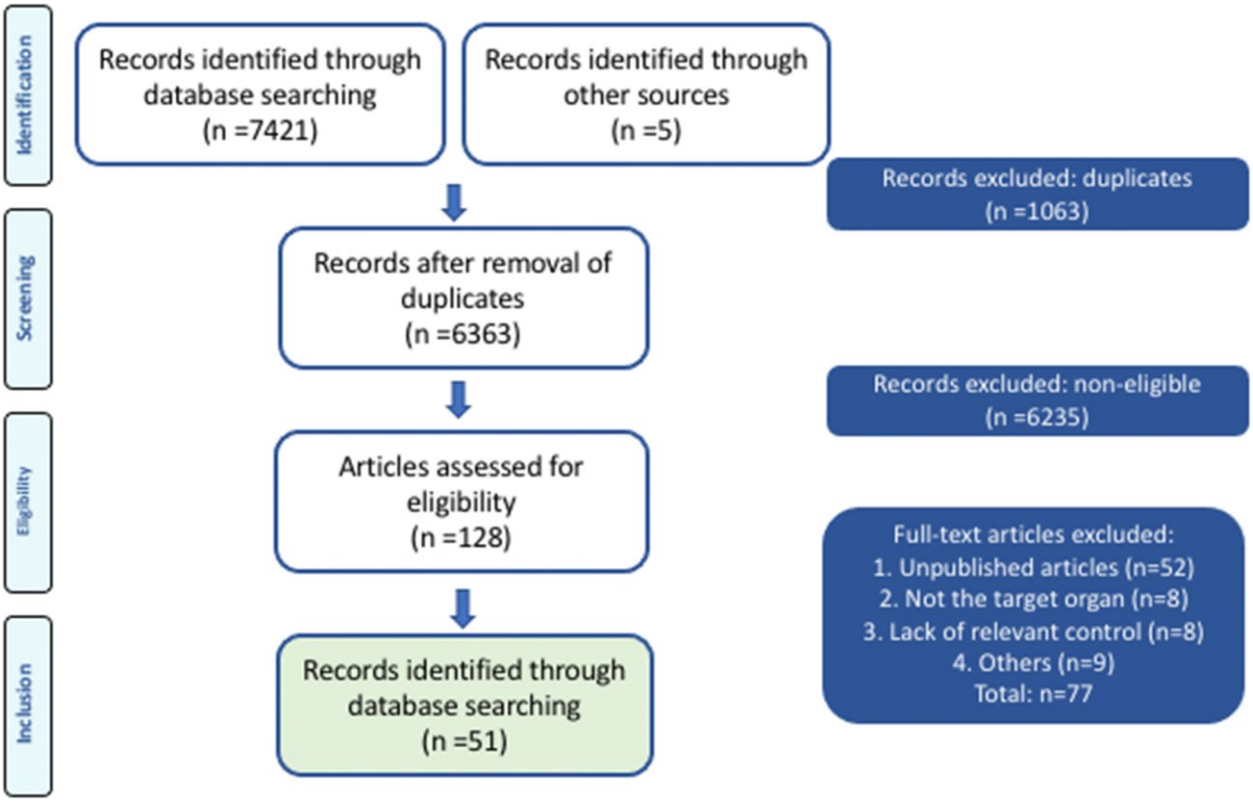

Figure 1. PRISMA flowchart depicting the pathway for selection of all included.

Table 1: SYRCLE's tool for assessing risk of bias. SYRCLE's risk of bias tool consists of a domain-based instrument with 10 items related to 6 types of bias: selection bias, performance bias, detection bias, attrition bias, reporting bias and other biases. These 10 items are organized in subitems in the form of questions that support a "Yes," "No," "Unclear answer." "Yes" refers to low bias with low risk; "No" refers to high bias with high risk; "unclear" is the degree of risk is uncertain. For easier orientation, answers at each domain is represented by colour dots as follows: • YES, • NO, • UNCLEAR

	Studies	**Type of bias and domains**									
		Selection bias			**Performance bias**		**Detection bias**		**Attrition bias**	**Reporting bias**	**Other**
		Sequence generation	*Baseline characteristics*	*Allocation concealment*	*Random housing*	*Blinding*	*Random outcome assessment*	*Blinding*	*Incomplete outcome data*	*Selective outcome reporting*	*Other sources of bias*
1	Alperin et al. (2010)[111]	•	•	•	•	•	•	•	•	•	•
2	Alperin et al. (2010) [112]	•	•	•	•	•	•	•	•	•	•
3	Alperin et al. (2015) [113]	•	•	•	•	•	•	•	•	•	•
4	Basha et al. (2013) [114]	•	•	•	•	•	•	•	•	•	•
5	Bracken et al. (2011) [71]	•	•	•	•	•	•	•	•	•	•
6	Callewaert et al. (2020)[115]	•	•	•	•	•	•	•	•	•	•
7	Catanzarite et al. (2018) [116]	•	•	•	•	•	•	•	•	•	•
8	Damaser et al. (2005) [117]	•	•	•	•	•	•	•	•	•	•
9	Daucher et al. (2007) [118]	•	•	•	•	•	•	•	•	•	•
10	Downing et al. (2013) [119]	•	•	•	•	•	•	•	•	•	•
11	Downing et al. (2014) [120]	•	•	•	•	•	•	•	•	•	•
12	Dhital et al. (2016) [121]	•	•	•	•	•	•	•	•	•	•
13	Emmerson et al. (2017) [97]	•	•	•	•	•	•	•	•	•	•
14	Ennen et al. (2011) [68]	•	•	•	•	•	•	•	•	•	•
15	Fajardo et al. (2008) [122]	•	•	•	•	•	•	•	•	•	•
16	Feola et al. (2010) [70]	•	•	•	•	•	•	•	•	•	•
17	Feola et al. (2011) [123]	•	•	•	•	•	•	•	•	•	•
18	Feola et al. (2014) [124]	•	•	•	•	•	•	•	•	•	•
19	Hympanova et al. (2019) [98]	•	•	•	•	•	•	•	•	•	•
20	Jackson et al. (2014) [69]	•	•	•	•	•	•	•	•	•	•
21	Jiang et al. (2014) [125]	•	•	•	•	•	•	•	•	•	•
22	Joyce et al. (2014) [126]	•	•	•	•	•	•	•	•	•	•
23	Kim et al. (2004) [127]	•	•	•	•	•	•	•	•	•	•
24	Knight et al. (2016) [128]	•	•	•	•	•	•	•	•	•	•
25	Kramer et al. (2006) [129]	•	•	•	•	•	•	•	•	•	•
26	Lemmex et al. (2016) [130]	•	•	•	•	•	•	•	•	•	•
27	Liang et al. (2016) [131]	•	•	•	•	•	•	•	•	•	•
28	Lindo et al. (2015) [132]	•	•	•	•	•	•	•	•	•	•
29	Lowder et al. (2007) [133]	•	•	•	•	•	•	•	•	•	•
30	Mao et al. (2019) [134]	•	•	•	•	•	•	•	•	•	•
31	Mattson et al. (2005) [135]	•	•	•	•	•	•	•	•	•	•
32	Moalli et al. (2008) [136]	•	•	•	•	•	•	•	•	•	•
33	Onol et al. (2006) [137]	•	•	•	•	•	•	•	•	•	•
34	Parkinson et al. (2016) [94]	•	•	•	•	•	•	•	•	•	•
35	Pierce et al. (2003) [103]	•	•	•	•	•	•	•	•	•	•

36	Pierce et al. (2007) [138]	🔴	🔴	🔴	🟡	🔴	🟡	🟢	🟢	🟢	🟢
37	Pierce et al. (2008) [139]	🔴	🔴	🔴	🟡	🔴	🟡	🟢	🟢	🟢	🟢
38	Rizk et al. (2007) [140]	🔴	🔴	🔴	🟡	🔴	🟡	🔴	🟢	🟢	🟢
39	Rizk et al. (2007) [141]	🔴	🟢	🔴	🟡	🔴	🟡	🔴	🟢	🟢	🟢
40	Ruano et al. (2011) [142]	🔴	🟢	🔴	🟡	🔴	🟡	🔴	🔴	🟢	🔴
41	Rynkevic et al. (2017) [96]	🔴	🔴	🔴	🟡	🔴	🟡	🔴	🟢	🟢	🟢
42	Rynkevic et al. (2019) [99]	🔴	🔴	🔴	🟡	🔴	🟡	🔴	🟢	🟢	🟢
43	Shveiky et al. (2019) [143]	🔴	🔴	🔴	🟡	🔴	🟡	🟡	🟢	🟢	🟢
44	Ulrich et al. (2014) [93]	🔴	🔴	🔴	🟡	🔴	🟡	🔴	🟢	🟢	🟢
45	Urbankova et al. (2019) [89]	🔴	🔴	🔴	🟡	🔴	🟡	🔴	🟢	🟢	🟢
46	Wieslander et al. (2008) [144]	🔴	🔴	🔴	🟡	🔴	🟡	🔴	🟢	🟢	🟢
47	Woo et al. (2007) [145]	🟡	🟢	🔴	🟡	🔴	🟡	🔴	🟢	🟢	🟢
48	Wood et al. (2008) [146]	🔴	🟢	🔴	🟡	🔴	🟡	🔴	🟢	🟢	🟢
49	Xelhuantzi et al. (2014) [147]	🔴	🔴	🔴	🟡	🔴	🟡	🔴	🟢	🟢	🟢
50	Young et al. (2017) [95]	🔴	🔴	🔴	🟡	🔴	🟡	🟢	🟢	🟢	🟢
51	Zong et al. (2009)[148]	🔴	🟢	🔴	🟡	🔴	🟡	🟢	🟢	🟢	🟢

Table 2: Summary of findings of active and passive biomechanical testing and structural analysis of the vagina in the different species used as a model for POP. Results are displayed as a comparison to nulliparous.

	Species	Passive Biomechanics	Active biomechanics	Collagen content	GAG content	Elastin content	Muscularis layer
Pregnancy	***Rat***	Increase compliance[123, 124, 133] and decrease tensile strength[123, 133]	Decreased contractility[123]	Decreased fiber aerea[118]	Decrease GAGs[142]	Upregulation of genes involved in the elastin metabolism[119]	—
	Sheep	Increase compliance and decrease tensile strength[92, 96, 99]	—	Decrease[92, 96, 99]	—	Increase[92, 96, 99]	Increase [92, 96, 99]
Spontaneous vaginal birth (Primiparous)	***Rat***	Initially increase compliance, which returned to normal 2w after delivery[120]	—	—	—	—	—
	Sheep	Increased compliance[89, 98, 128] and decrease tensile strength[128]	*Ambiguous* No difference[98] Decrease to KCl (distal vagina)[89]	Decreased[89, 98]	—	Increased [89, 98]	Decrease [89]
Spontaneous vaginal birth (Multiparous)	***Rat***	Increase compliance[120]	—	Decrease [121]	—	Tortuosity of the elastin fibers decreased [119]	Dissociation of collagen fibers with smooth muscle [121]
	Rabbit	—	—	Decrease[147]	—		Decrease[147]
	Sheep	*Ambiguous* No effect [99]or Increase compliance[94, 96, 97]	—	*Ambiguous* No difference [98]; decrease [96, 97, 99]or increase [92]	—	*Ambiguous* Decrease [92]or increase [96-98]	*Ambiguous* *No effect*[92]; Increase [99]or decrease[97]
	NHP	Increase compliance and decrease tensile strength[70]	—	Loss of collagen alignment[70]	—	—	—
SVD	***Rat***	Initially increase compliance, which returned to normal[119] increase compliance and decreased tensile strenght[111]	Initial hype- and later hyposensibility to carbachol (muscarinic receptors)[115]	No differences in connective tissue [115]and decrease collagen I/V ratio[111] on the long term	Increase [119],	Initial increased tortuosity of the elastin fibers, which normalized afterwards[119].	Decrease (disruption of smooth muscle layer) [111, 115]
Iatrogenic Menopause	***Rat***	Initially increase compliance and decrease tensile strenght[131, 136] and later decrease compliance[134]	*Ambiguous* for the vaginal region No effect in the proximal vagina. Distal vagina: decrease to KCl and phenylephrine.[137]	Initially no difference[131], later increase in the mature and decrease in the immature collagen [134]	No difference [131]	—	No difference [114] or Decrease[134, 137]

			Proximal vaginal: decrease to KCl[114]				
	Rabbit	Increased compliance in adults OVX but not adolescents OVX[130]	No difference[127]	Increase in adolescents OVX[130]	No difference [130]	—	Decrease [127]
	Sheep	No effect[98]	Decrease contractility in the middle vagina of OVX multiparous compared to intact multiparous [98]	Increase compared to intact multiparous [98]	—	Decrease only compared to intact multiparous [98]	No effect [98]
Aging	***Mouse***	—	—	—	—	Downregulation of genes involved in the elastin metabolism[125]	—
	Rat	No effect[136, 143]	—	—	—	—	—

Pregnancy and parity

Effects of pregnancy and parity on the vagina and the pelvic floor muscles was reported in 30 studies: one in mice [144], nine in rats [112, 113, 118, 120, 121, 123, 124, 133, 142] , two in rabbits [122, 147], eleven in sheep [68, 69, 89, 92, 94-99, 128] and seven in NHP [70, 71, 126, 129, 132, 135, 138] (Supplementary table 1). The effect of *pregnancy* was reported in 13 studies (mice=1 [144]; rats=7 [112, 113, 118, 123, 124, 133, 142] and sheep=5 [68, 69, 92, 96, 99]). Across species, pregnancy has a measurable effect, though that was most extensively documented in rats and sheep. Vaginal compliance increases during pregnancy [92, 96, 99, 123, 124, 133]. That is paralleled by microscopical and biochemical changes, i.e. a decreased collagen [92, 96, 99, 118, 142] and increased elastin [92, 96, 99]. The changes observed are considered as adaptations to prevent later damage caused by the passage of the fetus. The *first delivery* has an obvious effect. Most studies report an increase in compliance [89, 98, 128] and loss of tensile strength [128] of the vagina. Structurally, after the first delivery, collagen is reduced and elastin is increased [89, 98]. The additional effects of subsequent deliveries are variable and not similar in all studies. In some studies, in multiparous sheep, the initial effects of delivery actually recover [97, 98]. Available studies do not quantify the effect of age at the time of delivery, neither in rats nor in sheep.

***Denervation*:** Squirrel monkeys display POP under the form of cystocele following delivery, that is incremental with the number of deliveries. POP is facilitated by neurectomy, though neurectomy itself does not cause POP[103, 139] (Supplementary table 2).

Simulated vaginal delivery

Rats are the only species in which effects of delivery were studied by simulating this event (Supplementary table 3). Eight studies reported the effects on the vagina [111, 115, 117, 119, 120, 142, 145, 146] and one on the pelvic floor muscles [116]. SVD induces structural, active and passive biomechanical changes, which in rats partly heals and relatively fast, yet without complete recovery of the muscular layer [112, 115, 116], microvasculature [115] and biomechanics [112, 115]. However, SVD does not lead to POP.

***Iatrogenic Menopause*:** Changes induced by ovariectomy (OVX) in rats, rabbit and sheep were described in eleven studies [98, 114, 127, 130, 131, 134, 136, 137, 140, 141, 148] (Supplementary table 4). The effects depend on the age at what time OVX is done, and the interval to measurement. Menopause initially increases compliance [130, 131, 136], however later on the vagina becomes stiffer in rats [134]. No effect in sheep [98]. OVX induces atrophy of the vaginal epithelium [98, 127, 137], an increase in collagen [98, 130, 134] and decrease in elastin [98] and muscularis [127, 134, 137]. These effects are reversible by administration of hormones [114, 127, 131, 136, 141, 148]. OVX does not lead itself to prolapse.

Age

Five studies investigated the effect of aging on the vagina: one in mice [125], three in rats [136, 140, 143] and one in the baboon [135] (Supplementary table 5). All of them reported the effect of *natural* aging, except one study in mice, which reported as well the effect of a busulfan, a drug that accelerates aging. In mice and rats, there is no measurable biomechanical effect of age; however, there are arguments for a change in elastin metabolism [125].

Discussion and conclusion of findings in these studies

The effect of pregnancy and parity among species

Large animal models

Squirrel monkeys are non-human primates that are considered good models for studying the pathophysiology of pelvic floor dysfunction, including POP. Several risk factors for POP have been studied in this species, such as the relation with the pelvic outlet diameter, the age, parity and body weight [126]. Out of these, only *parity* was strongly correlated with the development of bladder descent (defined as 7 mm below the bony pelvis). The effects of pregnancy and delivery were studied in detail, using magnetic resonance imaging to assess the anatomy of the pelvic floor muscles, width of the bony pelvic outlet and measure bladder neck descent [71, 132]. The muscles studied were the levator, the obturator internus and coccygeus, which are all considered relevant to pelvic floor support. In particular, the coccygeus muscle was directly affected by the passage of the fetal head during delivery [71, 132]. Immediately postpartum, there is a *reduction* in levator and obturator internus volume, but this effect was *similar* following vaginal and abdominal delivery. The reduction would be the consequence of relative atrophy due to lesser physical activity of these muscle groups during pregnancy, hence not related to the delivery itself. Vaginal delivery was associated with a (temporary) increase in volume in the coccygeus muscle, that was not observed after cesarean section. This would be indicative of tissue edema, hence be an indirect sign of trauma by passage of the head. Eventually, at three to four months after delivery, there are no permanent anatomical changes in the pelvic floor muscles visible anymore [71, 132]. On the other hand, the bladder neck position is lower immediately after vaginal delivery and even more 3-4 months postpartum [71, 132] . This was associated with an increase in width of the pelvic outlet [132]. Remarkably, the extent of the descent and the width of pelvic outlet were similar 3-4 months postpartum, whether delivery was vaginal or by caesarean section [132]. The authors thought that this was due to permanent structural changes in the supportive pelvic floor ligaments and connective tissue induced during pregnancy (hence not birth). Therefore, in squirrel monkeys, cesarean section does *not* prevent changes induced by pregnancy and delivery. In another study, the presence of POP did not coincide with any gross anatomical differences in the pelvic floor muscles [129], but microscopically the myocytes of squirrel monkeys with POP were larger. No increase in apoptosis, disruption or atrophy were present [138]. We did not find information regarding the compliance and structural changes of the vagina in squirrel monkeys with POP.

Some, but not all primates, develop POP after (multiple) deliveries. For instance, multiparous Rhesus Macaques develop spontaneously descent of the cervix and posterior fornix – yet no other compartment [70], whereas multiparous baboons do not [135]. In the macaque, vaginal compliance increased and tensile strength became less, in analogy to what was described in sheep and rats [96, 97, 119]. Microscopically, the occurrence of POP coincided with a loss of collagen alignment but no difference in collagen subtypes.

Sheep are also said to develop "spontaneous" POP in the context of pregnancy and delivery [94, 95, 100]. They can develop impressive degrees of prolapse *before* birth. This suggests that, in some animals, structural effects occur during pregnancy, eventually leading to POP, though this may be a degree of laxity that is probably not what clinicians would consider as representative for what is a typical presentation in women. Both excessive weight gain during pregnancy, living on a steep terrain, as well as having twins (RR:5.0) and triplets (RR:11.0) are risk factors of *antepartum* POP [69]. In sheep with antepartum prolapse, there were no differences in progesterone or estradiol levels compared to those who did not [68]. At the gene expression level, sheep with antepartum POP display downregulation of collagen I [68], which is important in structural support. They also display hyperplasia of the vaginal epithelium, though they did not have elevated circulating estrogens [68]. The ewes with POP also had lower estrogen receptor alpha levels. This is different in premenopausal women with POP; who have been reported to have lower estrogen levels associated to less receptor expression [68].

Properties of pelvic floor structures can also be characterized by passive mechanical testing, which describes the relationship between stress and strain, and measurement of the disruption force. In sheep, pregnancy induces an increase in compliance and loss of tensile strength of the vagina [92, 96]. Other studies have documented the effects of a single or multiple delivery, compared to the status in virgins. Primiparous ewes have an increased vaginal compliance and lower tensile strength. Also, this seems to be the case for multiparous, except in two studies [98, 99]. One of these studies [95] is interesting, because it introduces a quantitative POP-system by measuring *spontaneous* displacement of a point 3 cm above the introitus, both anterior and posterior in the vagina, as well as a point above the urethra. Primiparous sheep displayed "displacement" in the lower areas, and multiparous also of the point higher up in the vagina. The authors concluded that sheep display "similar regions of weakness" as in humans [95]. In another study, the gross anatomic changes were documented in primi- and multiparous sheep. Primiparous sheep displayed an increased width and length of the vagina, which again returned to normal in multiparous sheep [98]. In another study in multiparous sheep, thinning of the vaginal wall was documented [97]. In conclusion, in sheep, pregnancy and delivery induce compliance and anatomical changes, but there is an inconsistency on whether multiple deliveries are causing incremental changes, and it remains unstudied whether changes observed after delivery, did occur during pregnancy and/or recover in between pregnancies. In future experiments, longitudinal observations should be included. This could be easily done with an elegant purposely designed device permitting non-destructive *in vivo* compliance measurements [97].

The findings at the microscopic level in sheep are less clear. *Pregnancy* induces an increase in elastin fibers, thickening of the muscularis layer, markedly less dense collagen in all vaginal layers as compared to virgin non-pregnant ewes, without change in vascularization in the lamina propria [92, 96, 99, 133]. The biochemical findings confirmed the increase in elastin but not the collagen changes [92]. The discrepancy between morphology and biochemical findings is not always easy to interpret. The long-term findings *after one or more deliveries* are quite conflicting. For instance, there are studies that did not document a change in collagen [97, 128] and others a decrease [96, 98, 99]. The same goes for elastin (increase in [96-99] and decrease in [92]) or the thickness of the muscularis (increased [96, 99] vs decreased [97]). One study also measured biochemically the total collagen, its subtypes, elastin, and glycosamin glycans (GAGs). The results do not all parallel the morphologic findings, making the interpretation not easy [92].

In conclusion, the overall impression is that, in sheep, *pregnancy* modifies the tissues such that the vagina becomes more compliant. This suggests that structural and functional adaptations may account for the ability of vagina to withstand "supraphysiologic" strains during parturition without injury[92, 96, 99]. Another method to study the mechanical properties of the vagina is by *active* contractility testing. Some consider this as a "functional" test. It is measuring the ability of the vaginal smooth muscle layer to contract, when exposed to agents like KCl or K^+ or by electric field stimulation. The response is proportional to the amount of smooth muscle tissue present. One can also stimulate the muscle via its innervation by adrenergic agents such as phenylephrine, epinephrine or norepinephrine, or by cholinergics like carbachol. Active biomechanical properties are only rarely reported in sheep. One study demonstrated no measurable long-term impact of parity on the active contractility of the vagina, not in primiparous neither in multiparous[98].

Smaller species

Lower species allow the study of pregnancy and delivery in much more detail, though as they are smaller the relevance of it may be more questionable. Again, all studies agree that *pregnancy* induces an increase in compliance and loss of tensile strength [92, 96, 99, 112, 123, 124, 133]. These changes recover after vaginal delivery, but the exact time point and level to which this occurs is inconsistent. In some this happens within one week [124], in others within four [123, 133], yet still incompletely [112]. In one study also multiparous animals were studied. They also display an increase in

compliance, compared to virgins. However virgin rats were 4m old and multiparous 9m-old [119]. Therefore, in that study it was not possible to conclude if the changes were due to parity or age, though other experiments have shown that age does not have an effect on vaginal biomechanics [136, 143]. The functional (active) response of vaginal smooth muscles during pregnancy and after delivery was tested as well. An increased sensibility to KCl was observed during late pregnancy, which did not return to pre-pregnancy values four weeks postpartum [123]. Another study investigated the function of the perineal and pelvic muscle following electric stimulation in rabbits. Multiparous had lower switch and tetanic tension force than virgin animals [122].

In rats, morphologic changes were also characterized. During late pregnancy the vagina lengthens, to normalize by four weeks postpartum [123]. On the other hand, the distal vagina is the widest at one week postpartum [124]. Moalli's group documented pregnancy induced microscopic changes, demonstrating an increase in the thickness of the muscularis layer, a decrease and loss of organization and orientation of the collagen fibers, and an increase in elastin [118]. Morphologically, the smooth muscle phenotype changes, from a quiescent to a proliferative and synthetic one [118]. Alperin's group described an increased muscle fiber length in the m. coccygeus, iliocaudalis and pubocaudalis in rats during late pregnancy [113]. This effect was associated to the adaptations of the PFM in order to have a protective effect against damage from large mechanical deformations likely occurring during parturition.

In one study, also GAG levels were assessed. GAGs play an important role in the properties of the extra-cellular matrix. In that study pregnancy induces a remarkable drop in GAG levels. Changes *after* delivery were documented in multiparous rats in two studies [119, 121]. There was a signature of collagen fiber dissociation with the smooth muscle and change in the density of collagen fibers [121]. In the study focusing on changes in vaginal GAG levels, a comparison in GAG levels after vaginal delivery and cesarean section was made. Vaginal delivery induces a deeper short-term drop in GAG levels than after abdominal delivery. On the long term (3 months), however, the GAG levels increase to far above pre-pregnancy levels. The study is unclear whether the difference between abdominal and vaginal delivered rats is significant [142]. The design of that study is an interesting one to dissect out the effects of pregnancy, delivery and the severity of birth trauma (the authors also simulated deliveries). Changes in the extracellular matrix metabolism were also studied in other species. For instance, in mice, a downregulation of Mmp2 and Mmp9 during pregnancy and immediately postpartum has been described reported [144].

Models that study the effects of vaginal birth

Denervation

Denervation, e.g. as in congenital birth defects such as spina bifida, or by traumatic delivery, has long been tied to the occurrence of POP. Denervation has been simulated in animal models, including for the study of POP. In rats, the effect of pudendal nerve crush has been widely studied, yet typically in the context of simulating urinary and fecal incontinence. Also experiments in squirrel monkeys involved denervation in the pudendal and LA nerve area. Atrophy of the m. pubocaudalis and iliocaudalis could only be induced by neurectomy of the LA nerve[103]. In other words, unlike in humans, the pudendal nerve does not innervate those muscles. The interesting part of that study was that the long-term effects of induced muscle atrophy were also documented [139]. Nulliparous squirrel monkeys that had undergone bilateral LA neurectomy did not develop bladder descent within two to three years. Apparently, despite muscle atrophy, other support structures prevent POP to develop. In the animals that had undergone neurectomy, who became pregnant, and delivered vaginally, all developed bladder descent. Two animals that died from obstetrical complications underwent necropsy, which revealed fibrosis, muscle atrophy and fatty replacement. In conclusion, denervation does not cause POP on itself, but may contribute to the onset of vaginal prolapse in animals that delivered vaginally. The effect of pregnancy or of cesarean section alone was not studied.

Simulated Vaginal Delivery

Researchers have also documented the effects of *simulated* vaginal delivery in rats, both on the vagina, pelvic floor anatomy as well as functional changes in vaginal function and urinary continence. Those experiments were conceived to provoke more changes than what is spontaneously occurring, since rats have a much smaller fetal head-to-pelvic outlet ratio than humans. One way to achieve this is by vaginal distention (VD). For that purpose, a balloon is inserted in the vagina and inflated with different volumes (2.5-5mL) and for a given duration (1-6hrs). This causes both mechanical stretch as well as hypoxia. Unfortunately, there is no standardization neither of the model, nor the read outs. The importance of standardization becomes obvious in an experiment made by the group of Alperin, et al. They documented the dose-response curve of increasing degrees of VD on the pelvic floor muscles (mm. coccygeus, ileocaudalis and pubocaudalis). They compared the effects to that of *spontaneous vaginal* delivery. The outcome measure of this experiment was the change in microstructure of the pelvic floor muscles (fiber and sarcomere length) in order to identify hyperelongation of sarcomere, as a primary cause of mechanical injury and resultant muscle dysfunction. A filling volume of 3mL distention mimics the effects of spontaneous vaginal delivery [116]. They demonstrated that delivery acutely stretches the myofibers, distorts the Z-lines and misalignment of adjacent sarcomeres, and increases sarcomere length. The changes were proportional to the distention volume used for simulation. The changes were also different when delivery was simulated in animals that were pregnant, versus rats that were not, i.e. that the injury was worse in the latter scenario. In other words, pregnancy has an attenuating effect on structural muscle changes, in particular in the m. coccygeus and m. pubocaudalis.

In two studies the changes in passive biomechanics of the vagina were investigated. VD increases vaginal compliance two days after injury, however normal properties were observed two weeks after delivery [119]. In another study with a higher filling volume, the increased compliance persisted up to four weeks after VD [111]. VD also induces anatomical changes. Macroscopically, the vagina was 20 to 50% wider following VD [111]. Microscopically VD causes a combination of hypoxia-induced and stretch injury in the vagina. Significant hypoxia in the epithelial layer and a lower level of hypoxia in the muscularis was observed one hour after VD [117]. VD induces a disruption of the fibromuscular layer of the vagina which persists until four weeks after delivery [111, 115]. On immunochemistry, VD induced an increase in collagen I/V ratio, but not in the I/III ratio, four weeks after delivery, and tortuosity of elastic fibers two days after VD [119]. By two weeks, the elastic fibers appeared normal [119]. As in spontaneous vaginal delivery, VD increase GAG levels in the vagina within 3 months, following an initial decrease 4d after VD [142]. In conclusion, VD induces extracellular matrix (ECM) production after the remodeling phase. Though immediately after VD there are no measurable differences in mRNA expression of genes related to inflammation or hypoxia [146], later on (by 24 hours) there is upregulation of MCP-3 and SDF-1, which are known markers of mobilization and homing of stem cells [145].

Another strategy is to induce nerve damage, which in rats typically is achieved by pudendal nerve crush (PNC). We could not find studies that document the effect of PNC alone on the vagina. The combination of VD and PNC creates longer lasting functional and anatomical effects. The downstream pelvic floor dysfunction effects can be measured at different levels, such as urethral and anal sphincter function, but also in the vagina. In several experiments, both strategies (VD and PNC) were *combined*, though only one focused on vaginal changes [115]. Active contractility was tested in the vagina at one, two, three and six weeks after VD+PNC. Functionally there seems to be a given time course, with an initial increased response to carbachol at two and three weeks and decrease at six weeks. The combination of VD and PNC induces a loss of microvasculature at one week, without recovery by six weeks. As following VD only, there was disruption of the muscle layer, which was eventually replaced by scar tissue. In the vagina, there was upregulation of smoothelin (smooth muscle regeneration), rock 1 (fibrosis) and muscarinic receptor 2 (acethylcholine receptor) at three days and downregulation of caldesmon (smooth muscle regeneration), and upregulation of collagen III at seven days. The authors concluded that initially the vagina has a hypersensitivity

denervation, which is characterized by an increase in the number of receptor sites, in an effort to maintain synaptic homeostasis, following neurotransmitter depletion. This was in agreement with the initial upregulation of muscarinic receptors 2 and its normalization later on, which coincided with a reduced sensitivity to carbachol. Another hypothesis was the impairment of the contractility by the process of fibrosis, since an increased collagen I/III ratio also took place 6 weeks after injury [115]. The initial upregulation of smoothelin may be seen as an attempt to regenerate the vaginal smooth muscle, however it seems that this process was impaired since a downregulation of caldesmon was also observed at 7 days.

Iatrogenic menopause

Ovariectomy *(OVX)* is the standard surgical procedure to investigate the effect of menopause in experimental animals, because the species used for the study of POP, do not develop spontaneous menopause. In rats, OVX reduces the stiffness of the vagina in young (four months) rats after eight weeks [131, 136], however no such effect was observed when OVX was performed in old (nine months) rats [136]. Another study reported a significantly increased stiffness 16 weeks after OVX in young rats [134]. In conclusion, on the longer term OVX in rats induces an increased stiffness of vaginal tissues. In rabbits, there was also an age dependent effect. In adult rabbits, OVX led to an increased compliance of the medial collateral ligaments (no measurements in the vagina were done), but not in adolescent rabbits [130]. In multiparous sheep, no change was seen160 d after OVX [98].

The currently available findings on active contractility are not consistent. In the distal [137] and proximal vagina of rats [114] and middle vagina of sheep [98] OVX decreased active contractility. This is not the case in rabbits [127].

Another outcome measure is vaginal morphology. Macroscopically OVX induces thinning of the vaginal wall in rats and rabbits [114, 127] and in sheep, the vagina gets shorter and narrower [98]. Atrophy of the epithelial layer is a consistent finding across species, including rats, rabbits and sheep [98, 114, 127, 134, 137]. This coincides with thinning of the layer of glycogen containing cells [98]. There is also atrophy of the muscularis [98, 127, 137]. One study in rats did not, but this may be due to the short interval between OVX and read out (3 weeks) [114]. An increase in collagen has been documented morphologically, both in rats and sheep [98, 134]; with, in rats, proportionally more mature collagen at 16 weeks [134]. This effect was not present in rats 8 weeks after OVX [131]. In sheep, a decrease in elastin was also reported [98]. At the protein level, OVX induces in rats upregulation of mature collagen and downregulation of immature collagen [134], and upregulation of a key collagenase (Mmp13) [148] and of urogenital aging markers (Isomyosin and $P27k^{ip1}$) [140]. Conversely downregulation of gene expression of muscle markers SM1 and caldesmon has been reported as well [114]. Hormonal replacement reverses most of the changes caused by OVX in rats, rabbits and sheep [114, 127, 131, 136, 141, 148].

Overall, the biomechanical response in experimental animals is dependent on the age when OVX is induced and the interval to measurement. On the longer term the vagina becomes stiffer, and this is associated an increase in collagen.

Aging

Both accelerated aging and natural aging led to a drop of 62% and 44% of estradiol compared to young mice, demonstrating an intertwinement between hormonal changes and advancing age [125]. Lower levels of estradiol were also observed in aging rats [143]. Both accelerated and natural aging induce downregulation of gene and protein expression of Lox3 and Lox4, which play a role on the synthesis of elastic fibers [125]. Again, in rats, aging did not influence the compliance of the vagina [136, 143]. However, aging seems to have an effect on the healing process of the vagina. Thirty days after injury, old rats regain only 15% of its original strength and compliance whereas young rats

recovered for 60%. This was associated to delayed and long-lasting expression of MIF (macrophage response). In baboons, aging did not coincide with more signs of POP [135].

Methodologic comment and recommendations

Most of the reviewed article had more than one methodological shortcoming. Most of studies missed proper animal randomisation and information on housing and if this was randomized. Animals differed at the baseline characteristic as weight, sex or age. Further, blinding of the researchers taking care of animals as well as researchers analysing the outcomes was mostly missing. Almost all studies did not use power calculation. Another problem is the heterogeneity of the methodology used, for example in the view of biomechanical testing, where broad spectrum of methodology is used. Future studies should avoid previously mentioned shortcomings by conducting well designed, powered and blinded studies with homogenous animal subject and methodology.

Conclusion

Several animal models have been used in the study the pathophysiology of POP, each with its own purpose, merits and limitations. In several species there are measurable effects of pregnancy, delivery and iatrogenic menopause, but there is not a single uniform pattern. Only squirrel monkeys develops clinical POP spontaneously.

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SUPPLEMENTARY DATA:

Supplementary table 1: Studies reporting the effect of pregnancy and parity in relevant species.

Reference	Sp. and relevant groups	N per group and time point	Tissue investigated	Outcome measures	Main effects
Wieslander, 2007 [144]	Mouse (age unknown); 1. Virgin, 2. Early pregnancy, 3. Late Pregnancy, 4. 2-48h PP, 5. 1w PP, 6. 2w PP	N=3	Vagina	Gene expression: Mmp2, Mmp9 and Mmp12 and their inhibitors	During pregnancy and immediately PP: downregulation of MMP2 and MMP9 and upregulation of elastase inhibitor; 48h PP upregulation of MMP2, MMP9,decrease of protease inhibitors.
Daucher, 2007 [118]	Rat (age unknown); 1. Virgin, 2. Mid pregnancy (12-14d), 3. Late pregnancy (20-22d), 4. Immediately PP 5. 3w PP	N=4	Vagina	Histology: Gomori Trichrome, Toluidine Blue TEM - morphology of smooth muscle cells and collagen fibril	Midpregnant and late pregnant: an increase in the number of papillae, the epithelium became mucified, an increase in matrix deposition in the subepithelium and muscularis Pregnancy decrease collagen fiber area and SMCs transforms from a quiescent and contractile SMCs to proliferative and synthetic phenotype. SMCs became diffusely spaced and surrounded by ECM. Immediately PP collagen fibers lost organization and orientation and the SMCs reduces the synthetic profile and normalized 3w PP.
Lowder et al., 2007 [133]	Rat (3m): 1. Virgin, 2. Mid pregnancy (d13), 3. Late pregnancy (19-21d), 4. Vaginal delivery (Immediately 0-4h and 4w), 5.CS (Immediately and 4w) - primigravid	N=8-13	Vagina and supportive tissue complex	Passive biomechanics	Pregnancy decrease stiffness and ultimate load at failure; maximal distension at the time of delivery; normalized at 4w PP
Alperin et al., 2010 [112]	Rat (age unknown); 1. Virgin, 2. Late pregnancy (17-20d), 3. 4w PP	N=7 -10	Vagina	In vivo passive biomechanics Vaginal dimensions (GH, TVL and vaginal diameter)	Compliance of the vagina increases during pregnancy and it recovered partially 4w PP but not until virgin compliance Genital hiatus, total vaginal length and vaginal diameters did not differ between the groups
Feola et al., 2011 [123]	Rat (3m); 1. Virgin, 2. Mid Pregnancy (14-16d), 3. Late Pregnancy (20-22d), 4. Immediately PP (0-2h), 5. 4w PP	N=6-8 for passive biomechanics and N=5-9 for active contractility	Vagina	Passive biomechanics Active contractility Vaginal dimensions (TVL and GH)	Decreased compliance during pregnancy and immediately postpartum, by 4 weeks postpartum recovered to virgin levels Contractile force decreased in mid- and late-pregnancy and immediately post- partum compared to virgin animals, 4 weeks postpartum returned to virgin levels TVL and GH values increased from virgin to late-pregnant animals and immediately PP; values normalized 4w PP

Ruano, 2011 [142]	Rat (4m); 1. Virgin, 2. Virgin SVD, 3. CS, 4. CS + SVD, 5. Spontaneous vaginal delivery, 6. Late pregnancy (d20)	N=10	Vagina, 4d or 3m after delivery or SVD	GAG biochemical analysis	Pregnancy decrease the levels of GAGs; normal delivery or SVD increase GAGs at 3m
Downing, 2013 [119]	Rat (age unknown); 1. Virgin, 2. Virgin SVD 3. Spontaneous delivery, 4. CS; SVD: VD 2.5ml, hanging catheter for 3h; still inflated catheter when removed	N=5-6	Vagina, 2d and 2w after delivery or SVD	Gene expression (genes involved in elastic fiber homeostasis): Loxl-1, Fbln5, tropoelastin	Vaginal delivery, SVD and pregnancy cause upregulation of genes involved in elastic fiber homeostasis.
Downing, 2013 [120]	Rats (age unknown); 1. Virgin, 2. Virgin SVD, 3. Vaginal delivery, 4. SC, 5. Multiparous	N=6 per group and time point	Vagina, 2d and 2w after delivery or SVD	Passive Biomechanics	Regardless the mode of delivery, vagina have a significantly higher compliance 2d after delivery compared to virgin rats; it normalizes at 2w. In multiparous compliance was higher than in nulliparous.
				Histology - Hart s - Morphometric analysis of elastin fibers	Regardless the mode of delivery, vagina have a significantly higher tortuosity of elastic fibers 2d after delivery compared to virgin rats; and it normalizes at 2w. In multiparous tortuosity of elastic fibers was higher than in nulliparous.
Feola et al., 2014 [124]	Rat (age unknown); 1. Virgin, 2. Longitudinal study: Pregnancy (15d and 18d) and PP (7d, 30d)	N=6-8	Vagina	In vivo biomechanics of the vagina by 3D US	Pregnancy increase compliance (3.5x at 15d and 5.4x at 18d; it normalizes at 1w PP
				Shape and size of vagina	Distal vagina has the highest difference in the cross-sectional area; which 1w PP had the largest area (58% larger than 4w PP)
Alperin, 2015 [113]	Rats (3m); 1. Young Virgin (3m), 2. Mid pregnancy 3. Late pregnancy, 4. Mature Virgin (6m) ,5. 4w PP, 6. 12w PP	N=10	PFM	Histology: Morphometric analysis (fiber and sarcomere length and physiologic cross-sectional area)	Late pregnancy induces increase of fiber length in all PFM (coccygeus, iliocaudalis and pubocaudalis), no effect in tibialis. Recovery of fiber length at 12w PP. No effect in sarcomere length and cross-sectional area;
				Total collagen	Increased during pregnancy, no decrease in coccygeus PP.
Dhital, 2016 [121]	Rats; 1. Virgin (11–14 w), 2. Multiparous (9–15 m), 3. 2d PP (primiparous), 4. 14d PP (primiparous)	N=4	Vagina	Analysis of collagen by nuclear magnetic resonance (NMR)	Virgin rat tissues contained higher collagen content compared to the other three groups
				Biochemical analysis of collagen	Multiparous rats showed higher collagen content values compared to 2 and 14 d PP
				Histology: Van Gieson	Collagen fiber dissociation with smooth muscles in multiparous rats

Fajardo, 2008 [122]	Rabbit (10-14m); 1. Virgin, 2. Multiparous	N=5	Perineal and Pelvic Muscles (Bulbospongiosus, Pubococcygeus)	Histology: Sudan Black Contractile properties - electrical stimulation Weight of muscle	Multiparous have smaller cross-sectional muscle fiber area Multiparous have lower switch and tetanic tension force in response to electric stimulation Multiparous have lighter muscle
Xelhuantzi, 2014 [147]	Rabbit; 1. Virgin, 2. Multiparous (4 deliveries); Subgroups: young (9-11m) and adult (17-18m)	N=6	Vagina	Histology: MT	Multiparous: Disorganization of the tissue components in vagina; less collagen, muscle and vessels
Ennen et al., 2011 [68]	Sheep (3.2 ± 1.4 y); 1. Healthy pregnant, 2. Intrapartum, 3. PP with POP	N=4-6	Blood	Radioimmunoassay: Serum levels of Progesterone and 17β-Estradiol	No difference
			Vagina	Histology: HE	POP: hyperplasia of the epithelial cells. No evidence of increased inflammation.
				Gene expression RT-PCR: GAPDH, Mmp1, Timp1, ER-α, Col1a2	POP: Downregulation of col1a2 and ER-α
Jackson et al., 2014 [69]	Sheep (mixed age): 1. Whole farm population, 2. Randomly selected ewes (2y and older)	N=36,695	-	Epidemiologic test; risk factors of vaginal prolapse	5x higher chance of POP with twins; 11x triplets; higher risk with weigh gain during pregnancy, access to salt and steep terrain
Ulrich, 2014 [92]	Sheep; 1. Virgin (1y), 2. Late pregnancy (with 3 previous deliveries; 4-5y), 3. Multiparous (3 deliveries; 4-5y)	N=3-6	Vagina	POPq	Virgin and parous no displacement. In pregnancy, the mean displacement was 3.3 cm with traction of both vaginal walls.
				Passive Biomechanics	Pregnant: weakest and most compliant vagina; parous: highest max stress; virgin: strongest and still compliant tissue
				Biochemical analysis (hydroxyproline (Hyp) assay :total collagen, SDS-polyacrylamide gel electrophoresis: collagen III/I, colorimetric dimethylmethylene blue (DMMB) assay : GAG and indirect insoluble elastic tissue associated proteins (ETAP) analysis: elastin)	Parous increase in collagen and decrease in elastin compared to virgin;. Pregnant increase elastin compared to virgin. Similar levels of total collagen. No difference in collagen III/I. The virgin lower GAG in the anterior wall compared to the parous.
				Histology: HE and MT, Verhoff Van Gieson	Pregnant showed fewer cells but similar density of blood vessels in the lamina propria and markedly less dense tissue and collagen packing in all layers compared to virgin. Parous had the vaginal architecture similar to that of virgin sheep (epithelial height, vessel density, densely packed collagen). Parous less elastin fibers than virgin.
				IHC for α-SMA	Pregnant had the highest proportion of muscularis; and it was significant more than virgin sheep. No significant difference between parous and virgin.
Parkinson, 2016 [94]	Sheep; 1. Virgin (2y), 2. Parous (1-4 deliveries, 3-5y)	N=3-15	Vagina	In vivo vaginal pressure	Higher compliance in the parous vagina; mainly at the cervical region

Knight, 2016 [128]	Sheep (2.5 - 3.5y); 1. Virgin, 2. Primiparous	N=7-8	Vagina	POP-Q	Shorter perineal body and greater anterior and posterior vaginal wall descent in the parous relative to nulliparous
				Passive biomechanics	Virgin had 51% stiffer and 36% stronger than parous;
				Total collagen: Hydroxyproline Assay	No difference
				Histology: MT, picrosirius red	No difference in collagen fiber thickness
Emmerson, 2017 [97]	Sheep; 1. Virgin (2y), 2. primiparous (3-4y), 3. Multiparous (4-5y)	N=6-8	Vagina	POP-Q, Pressure sensor	Multiparous significantly more vaginal displacement at all 3 "POP-Q" points compared to nulliparous
					Multiparous vagina exerted lower pressure than virgin
				Passive biomechanics	Multiparous weaker and more compliant than virgin
				Histology: HE, MT, Hart´s elastic Picro-Sirius Red IHC: α SMA	No difference in collagen organization; Multiparous has thinner muscularis, primiparous and multiparous increased elastic fibers compare to nulliparous.
				Total collagen: Hydroxyproline Assay	Primiparous significantly less collagen compares to nulliparous
Rynkevic et al., 2017 [96]	Sheep; 1. Virgin (9m), 2. Pregnant (2 previous deliveries, 3y) 3. Multiparous (1y after 3rd delivery, 4y)	N=5	Vagina	Passive Biomechanics	Pregnant sheep 64% more compliant vagina than virgin and 47% more than multiparous; Parous sheep has weaker vagina than virgin
				Histology: Miller's Elastica	Pregnant and parous have less collagen and more elastin and smooth muscle than virgin.
Young et a., 2017 [95]	Sheep; 1. Virgin (2y), 2. Multiparous (1-4 delivery, 3-5y), 3. Multiparous (no birthing history, 6y)	N=14-56	Vagina	POP-Q	Ovine vaginal displacement was seen in 50.9 % of parous ewes and was strongly associated with parity. Nulliparous: minimal vaginal wall displacement.
Hympanova et al., 2019 [98]	Sheep; 1. Neonatal (1d), 2. Prepubescence (0.3y), 3. nulliparous (1y), 4. Primiparous (1y PP, 2y), 5. Multiparous (7y), 6. menopausal (7y)	N=6	Vagina	Passive biomechanics	Distal vagina increase compliance in primiparous, this is reversed later.
				Active contractility	No difference
				Vagina size	Increase in width and length in primiparous, decreasing in multiparous.
				Histology: HE, PAS, MT, Miller's Elastica	Increased epithelial thickness in multiparous. Decrease collagen and increased elastin after first and multiple deliveries.
Rynkevic et al., 2019 [99]	Sheep; 1. Virgin (9m), 2. Pregnant (3y, 2 previous deliveries), 3. Multiparous (4y, 3 deliveries)	N=5	Vagina cervix, uterus, bladder, rectum, and muscles (external anal sphincter and levator ani muscle)	Passive biomechanics	Vaginal wall and cervix more compliant in pregnant compared to virgin; no difference between virgin and multiparous
				Histology: Miller	Pregnant vagina has less collagen, more elastin and more smooth muscle than virgin; Multiparous has less collagen, more elastin and more smooth muscle than virgin.
		N=6	Vagina	Passive Biomechanics	Primiparous: Increased compliance in the distal vagina;

Urbankova et al., 2019 [89]	Sheep (1-3.5y); 1. Virgin, 2. Primiparous (1 PP), 3. Multiparous after OVX, 4. Multiparous after OVX with HRT			Active contractility Dimensions Histology: HE, PAS, MT, Miller's pentachrome IHC for α-SMA and ER-α	Primiparous: decrease in 64% de SM contraction to KCl in the distal vagina; Primiparous: wider and longer vagina Primiparous: 79% more elastin and 29% less collagen only in the distal vagina. Primiparous: Lower expression of ER-α in the epithelium of the distal vagina; tendency to decrease α-SMA expression in the distal vagina.
Mattsom et al., 2004 [135]	Baboon; 1. Virgin (4.8y) 2. Multiparous (23y)	N=6	Vagina	POP-Q	Multiparous showed no evidence of POP or differences in POP-Q from nulliparous
Feola et al., 2010 [70]	Rhesus Macaques (9-19y); 1.Virgin, 2. parous (minimum 1y PP)	N=7	Vagina	POP-Q Passive biomechanics Histology: MT, Sirius red IHC: collagen I,III,V	Greater descent in parous: shorter distance Hymen-cervix and Hymen-fornix Increased compliance and decrease tensile strength in parous Loss of collagen alignment in parous but not difference in collagen subtypes
Kramer et al., 2006 [129]	Squirrel monkey (parous); 1. POP (13y) 2. non-POP (15y)	N=7	PFM (LAM and obturator internus)	MRI – muscle volumes Muscle volumes and weights from necropsy after MRI	Levator ani volumes were higher in parous with POP, obturator internus did not differ.
Pierce et al., 2007 [138]	Squirrel monkey, 1. Virgin (9y), 2. Parous non-POP (17y), 3. Parous POP (13y)	N=4-7	PFM (LAM and paravaginal attachments)	Histology: HE, MT, Van Gieson IHC: for WGA-TRITC, My-32 and tunnel	Signs of myogenic damage were found more often in the pubocaudalis muscle than the iliocaudalis, yet did not differ between POP and non- POP Increased diameter of myocyte in POP; no increase in apoptosis; no disruption or atrophy of LAM
Bracken, 2011[71]	Squirrel Monkeys (nulliparous, 4-5y); longitudinal evaluation 1. non pregnant 2. 3d PP 3. 4m PP	N=8	PFM (LAM, obturator internus, and COC)	MRI: volume of PFM and bladder neck and cervix position	Only COC increased volume after delivery but it recovers. Parity lead to descent of bladder neck and cervix without recovery.
Joyce et al., 2014 [126]	Squirrel monkey (parous); 1. POP (12y) 2. non-POP (9y)	N=19-36	-	MRI: Correlation of POP with pelvic outlet diameter, age, parity and weight	Only parity shows a strong correlation with POP. Pelvic outlet diameter size does not contribute to POP.
Lindo, 2015 [132]	Squirrel Monkey (nulliparous, 3.9y); longitudinal valuation 1. VD 2. CS Examination prior pregnancy, 1-5d after delivery, 3m after delivery	N=10	PFM (LAM, obturator internus, and COC)	MRI: muscle volumes and bladder neck position	Volume of LAM and obturator internus did not differ between groups and decreases immediately after pregnancy; COC increase only after VD; Bladder neck descends by 3m postpartum in both groups.

POP: pelvic organ prolapse; POP-Q: pelvic organ prolapse quantification system, PFM: pelvic floor muscle; MRI: magnetic resonance imaging; MT: Masson's Trichrome; PAS: periodic acid shiff, IHC: immunohistochemistry; LAM: levator ani muscle; HE: Hematoxylin and Eosin; GAPDH: glyceraldehyde 3-phosphate dehydrogenase, Mmp1: metalloproteinase 1, Timp1:inhibitor of metalloproteinases 1; Col1a2: collagen I; WGA: wheat Germ Agglutinin - Myocyte; My-32: fast skeletal myosin heavy chain; ER- α: estrogen receptor alpha; COC: coccygeus muscle, PP: post-partum, CS: cesarean section,

VD: vaginal delivery, PAS: periodic acid-Schiff. Y: year, d: day, w: week, h: hour; TEM: transmission electron microscopy, SVD: simulated vaginal delivery, GAGs: glycosaminoglycans, GH: genital hiatus, TVL: Total vaginal length, OVX: ovariectomy, HRT: hormonal replacement therapy; SM: smooth muscle

Supplementary table 2: Studies reporting the effect of denervation on pelvic floor muscles in relevant species. Only studies on squirrel monkeys were found.

Reference	Species and groups	N per group and time point	Tissue and Time Point	Outcome measures	Main effects
Pierce et al., 2003 [103]	Squirrel Monkey (nulliparous, 2-3 y, one 14y); Neurectomy of pudendal or LAM nerve.	N=2-4	PFM, 2 w after neurectomy	Gross anatomy	Intrapelvic anatomy is similar to humans, but pudendal nerve does not innervate PFM, as in humans
				IHC for WGA	Only LAM neurectomy lead to muscle atrophy
				Koele Staining (motor endplate)	One endplate zone per muscle
				Muscle Mass and myocyte size	Only LAM neurectomy lead to loss of mass of pubocaudalis and iliocaudalis
Pierce et al., 2008 [139]	Squirrel Monkey (2 to 25 y); 1. Virgin non-POP (5.9y), 2. Parous non-POP (15.4y), 3. Parous POP (11.1y), 4. Bilateral neurectomy of LA (5y);	N=6-17	LAM, 2-3y after neurectomy PFM, 2-3y after neurectomy	MRI with or without abdominal pressure (volume of LA and position of bladder and cervix)	LAM Denervation caused a decrease in the LAM volume; It also seems to accelerate the descent of bladder after parturition (57% of the cases after 2nd delivery)
				Gross anatomy	Intrapelvic anatomy is similar to humans, but pudendal nerve does not innervate PFM, as in humans

POP: pelvic floor prolapse; PFM: pelvic floor muscle; MRI: magnetic resonance imaging; LAM: levator ani muscle; WGA: wheat Germ Agglutinin – Myocyte, IHC: immunohistochemistry

Supplementary table 3: Studies reporting effects of simulated vaginal birth.

Reference	Species and groups	N per group and time point	Tissue investigated and time points	Outcome measures	Main effects
Damaser, 2005 [117]	Rat (age unknown); 1. Virgin, 2. Virgin SVD (VD 3ml for 1h)	N=4-5 per group and time point.	Vagina, immediately before, immediately after, 15min and 1h after SVD	Vascular perfusion by Microspheres	Decrease perfusion in the vagina at 0 min but it recovered at 5 min
				IHC for Hypoxiprobe	Significant hypoxia of the of the stratum spinosum of the vaginal epithelium and low level of hypoxia in the muscularis
Woo, 2007 [145]	Rat (age unknown); 1. Virgin sham (only anesthesia), 2. Virgin controls (no interventions), 3. Virgin SVD (VD 3ml for 4h)	N=4	Vagina, 0h and 24h after SVD	Gene expression: MCP-3 and SDF-1	Upregulation of MCP-3 immediately and 24h after VD and downregulation of SDF1 immediately after VD.
Wood, 2008 [146]	Rat (10w); 1. Virgin sham, 2. Virgin SVD (VD 3ml for 1, 4 and 6h)	N=5	Vagina, 0h after SVD	ression: SDF-1, CXCR-4, CCR 1, CCR-2, CCR-3, CCR-5, VEGF, MCP-3, Il-8, HIF--1α	No difference at any time duration of VD
Alperin, 2010 [111]	Rat (3m); 1. Virgin, 2. Virgin SVD (VD 5ml, hanging 130g for 2h)	N=10 for histology and n=8 for passive biomechanics	Vagina, 4w after SVD	Passive biomechanics	Increase compliance, decrease tensile strength after SVD
				Histology: MT	Disruption of fibromuscular layer
				IHC for collagen subtypes I, III, V	Decrease in I/V collagen ratio after SVD; no difference in I/III ratio
				Size of vagina (genital hiatus (GH) and total vaginal length (TVL))	1.2-1.5-fold increase in diameter
Ruano, 2011 [142]	Rat; 1. Virgin, 2. Virgin SVD, 3. CS, 4. CS + SVD, 5. Spontaneous delivery, 6. Late pregnancy (d20); SVD: VD 5ml, hanging 100g for 3h	N=10	Vagina, 4d or 3m after delivery or SVD	GAG biochemical analysis	Pregnancy and spontaneous delivery decrease the levels of GAGs at 4d; spontaneous delivery or SVD increase GAGs at 3m
Downing, 2013 [119]	Rat (age unknown); 1. Virgin, 2. Virgin SVD 3. Spontaneous delivery, 4. CS; SVD: VD 2.5ml, hanging catheter for 3h; still inflated catheter when removed	N=5-6	Vagina, 2d and 2w after delivery or SVD	Gene expression (genes involved in elastic fiber homeostasis): Loxl-1, Fbln5, tropoelastin	Vaginal delivery, SVD and pregnancy cause upregulation of genes involved in elastic fiber homeostasis.
Downing, 2013 [120]	Rats; 1. Virgin (12-15w), 2. Virgin SVD, 3.Spontaneous delivery, 4. CS, 5. Multiparous (9-12m); SVD: VD 2.5ml, hanging catheter for 3h; still inflated catheter when removed	N=6	Vagina, 2d and 2w after delivery or SVD	Passive Biomechanics (Pressure-infusion system)	SVD at 2d and Multiparous vagina have a higher compliance than virgin; it normalizes at 2w.
				Histology: Van Giesen – Tortuosity Hart s - Morphometric analysis of elastin fibers	2 days after delivery, SVD and in Multiparous significantly higher tortuosity of elastic fibers compare to virgin; at 2w virgin, SVD, spontaneous delivery and CS showed similar elastic fibers
Catanzarite, 2018 [116]	Rats (3m); 1. Virgin control, 2. Virgin SVD, 3. Pregnant-late control (20-21d), 4. Pregnant SVD, 5. Intrapartum (spontaneous delivery); SVD: VD 1-5ml, hanging 130g for 1-2h	N=10-22	PFM, 0h after SVD	Fiber and sarcomere length of PFM	SVD causes acute and progressive stretch of the myofibers at higher distention volumes, mainly coccygeus and pubocaudalis. At 3ml mimics the spontaneous vaginal delivery. SVD in virgin causes a significant longer sarcomere length compared to pregnancy
				Transmission electron microscopy (TEM)	SVD causes distortion of Z-lines and misalignment of adjacent sarcomeres mainly in Coccygeus and pubocaudalis

				Genital hiatus, total vaginal length, and perineal body	Genital hiatus increased during vaginal distention in both nonpregnant and pregnant
		N=6 except for PCR (n=5)		IHC for CD34, α-SMA, PPG9.5	SVD cause rupture of smooth muscle layer with scar deposition; Loss of microvasculature at 1w and 6w; no difference in PPG9.5 expression
Callewaert, 2020 [115]	Rats (12 to 15 w); 1. Virgin control, 2. Virgin SVD; SVD: PNC + VD 3ml for 4h		Vagina, 3d and 1, 2, 3 and 6w after SVD	Active contractility	Hypersensitivity to carbachol at 2 and 3w after SVD; Hyposensitivity at 6w
				Gene expression: caldesmon, muscarinic receptor 2 and 3, smoothtelin, rock 1, col1a1, Col3a2,	At 3d: upregulation of smoothelin, rock 1 and muscarinic receptor 2; at 1w: downregulation of caldesmon and upregulation of collagen III

SVD: simulated vaginal birth; VD: Vaginal distention; PNC: pudendal nerve crush; α-SMA: smooth muscle actin; MT: Masson`s Trichrome; PFM: pelvic floor muscle; MCP-3: Monocyte chemotactic protein-3; SDF-1: Stromal derived factor-1; VEGF: vascular endothelial growth factor; FBLN5: Fibulin 5; CS: cesarean section h: hour, IHC: immunohistochemistry, min.: minute

Supplementary table 4: Studies reporting the effect of iatrogenic menopause in relevant species

Reference	**Sp. And Groups**	**N per group and time point**	**Tissue investigated and time points**	**Outcome measures**	**Main effects**
		N=15			OVX decrease contractility only of distal vagina to EFS and decreased response to phenylephrine and α -1 and 2 blockade; no difference to carbachol. No effect on the proximal vagina
Önol et al., 2006 [137]	Rats (6m); 1. Intact mature, 2 OVX mature.		Vagina, 6w after OVX	Active contractility	
				Histology	OVX caused smooth muscle and epithelium layer atrophy.
				TEM	OVX induced severe degeneration of epithelial layer
Rizk et al, 2007 [140]	Rats; 1. Intact young (3m), 2. OVX young (3m), 3. Intact old (18m), 4. OVX old (18m)	N=6	LAM, 4w after OVX	WB: Isomyosin and $p27^{kip1}$	OVX increased isomyosin in old rats and $p27^{kip1}$ in both young and old rats
Rizk et al, 2007 [141]	Rats; 1. Intact old (13m), 2. OVX old, 3.OVX+E2, 4. OVX ghrelin, 5. OVX E2+ghrelin; treatment started 4w after OVX	N=6	LAM, 10w after OVX	WB: $p27^{kip1}$	OVX increased 44% of $p27^{kip1}$; estrogen restored the normal levels and ghrelin decreases to below sham levels
Moalli et al., 2008 [136]	Rats; 1. virgin young (4m), 2. Parous Young (4m), 3. Parous middle age (9m); all with subgroups: a. intact, b. OVX, c.OVX+CMT8, d. OVX+E2, e. OVX+E2+P4	N=34-40	Vagina, 8w after OVX	Passive biomechanics	Increased compliance and decrease ultimate load after OVX, only in young rats; CMT8, E2 and E2+P4 restored tissue quality.
Zong et al., 2009 [148]	Rats (3m); 1. Intact, 2. OVX, 3. OVX E2+P4	N=6-9	Vagina, 8w after OVX	WB: Mmp13	OVX increase activity of Mmp13; this was suppressed by E2 + P4 supplementation
		N =3 for histology; n=5 for active contractility and protein analysis; n=6 for estradiol		Active contractility of the proximal vagina	OVX decrease contractility to KCL, E2 reverse the effect
				Serum Estradiol	OVX decrease estradiol levels
Basha et al., 2013 [114]	Rat (3-4m); 1. Intact, 2.OVX, 3. OVX+E2 (Starting 2w after OVX)		Vagina, 3w after OVX	Gene expression: MHC isoforms, carboxyl terminal myosin heavy chain and caldesmon	OVX downregulate %SM1 in MHC, E2 reverse the effect.
				WB: caldesmon and MHC	Decrease in caldesmon (2x); E2 reverse the effect; no effect in MHC

				Coomasi staining of carboxyl terminal isoforms	Decrease in SM1, not SM2, E2 reverse the effect.
				Histology (MT)	OVX decrease epithelial thickness and leads to thinning of the vaginal wall. No difference in the muscularis. E2 reverse the effect
Liang et al., 2016 [131]	Rat (4m); 1. Intact, 2. OVX, 3. OVX+E2	N=8	Vagina, 8w after OVX	Passive Biomechanics	OVX cause increase compliance (34%) and decrease tensile strength (16%). E2 restored to intact levels.
				Biochemistry (total collagen and GAG)	No difference between the groups
		N=6		Passive biomechanics	OVX caused decreased compliance and decreased ultimate load at 16w
Mao et al., 2019 [134]	Rat (8w); 1. Intact young, 2. OVX		Vagina; 2, 4 and 16w after OVX	WB: Col1 and Col3	OVX increased col1 and decreased col3 at 2w
				IHC for α-SMA	OVX decrease the fraction of α-SMA and smooth muscle bundles become disorganized at 2w
				Histology (HE and sirius red)	Atrophy of epithelium at 16w; Decrease in immature collagen and increase in mature collagen at 16w
		N=4-7		Active contractility	No difference
Kim et al., 2004 [127]	Rabbit (age unknown); 1. Intact, 2. OVX, 3. OVX+E2, 4.OVX + testosterone		Vagina, 4w after OVX	Histology	OVX: thinning of vaginal wall, atrophy of vaginal epithelium and muscularis; E2 restore epithelium but only partially the muscularis; Testosterone restore muscularis but only partially epithelium.
		N=7-8		Passive Biomechanics	OVX leads to higher compliance in adults but not adolescents, which showed only higher strain
Lemmex et al., 2016 [130]	Rabbit; 1. intact adult (1y), 2. OVX adult (1y14w) 3. OVX adolescent (48w)		Medial collateral ligaments; 14w after OVX (adult) and 33w after OVX (adolescent)	Biochemistry (collagen and GAG)	Only adult showed higher total collagen
				Gene expression: Biglycan, Col-I, Col-III, Col-V, decorin, estrogen receptor, Mmp1, Mmp3, Mmp13, Progesterone receptor, Lubricin, Timp1, Timp2 Timp3	Upregulation of lubricin and col1 in adults; downregulation of progesterone receptor in adolescents
		N=6		Passive biomechanics	No difference
Hympanova, et al. 2019 [98]	Sheep (7y); 1. Intact Multiparous 2. OVX Multiparous		Vagina, 160d after OVX	Active contractility	No difference in the distal vagina; Middle vagina lower contractility to KCl
				Vagina size	OVX shorter and narrower vagina
				Histology	OVX higher collagen and lower elastin; OVX sheep had less glycogen and epithelium atrophy in both distal and middle vagina

OVX: ovariectomy; EFS: electro field stimulation; GAG: glycosaminoglicans: MHC: Myosin Heavy Chain; α-SMA: smooth muscle actin; E2: estrogen (estradiol); P4: progesterone; CMT-8: chemically modified tetracycline-8 (matrix metalloproteinase inhibitor), WB: western blot, m: month, w: week, y: year; LAM: levator ani muscle; Mmp13: matrix metalloproteinase 13, MT: Masson`s Trichrome, HE: Hematoxylin & eosin, Col: collagen

Supplementary table 5. Studies reporting the effect of aging in relevant species

Reference	Species and groups	N per group and time point	Tissue investigated and time points	Outcome measures	Main effects
Jiang et al., 2014 [125]	Mouse; 1. Young (3m), 2. Young accelerated aging model (3m; Busulfan), 3. Old (18m)	N=20, except for blood samples (n=3-4)	Vagina, 1 m after Busulfan (accelerated aging model)	Serum Estradiol Gene expression: elastin, Lox, (Lox 1, 2, 3, 4) WB: Lox 3, Lox 4	Accelerated aging leads to reduction in 62% of estradiol while aging led to 44% reduction Downregulation of all LOX family and elastin in both aging and accelerated aging Downregulation of LOX3, Lox4 in both aging and accelerated aging
Rizk et al, 2007 [140]	Rats; 1. Intact young (3m), 2. OVX young (3m), 3. Intact old (18m), 4. OVX old (18m)	N=6	LAM, 4w after OVX	WB: Isomyosin and p27kip1	Aging increased both isomyosin and p27kip1 in old rats
Moalli et al., 2008 [136]	Rats; 1. virgin young (4m), 2. Parous Young (4m), 3. Parous middle age (9m); all with subgroups: a. intact, b. OVX, c.OVX+CMT8, d. OVX+E2, e. OVX+E2+P4	N=34-40	Vagina	Passive Biomechanics	No difference related to aging, only OVX
Shveirky et al., 2019 [143]	Rats; 1. Young (3m), 2. Old (12m)	N=5	Vagina	Passive Biomechanics 30d Wound healing 1, 3, 7, 14d post injury Gene expression: MIF at 30d Estradiol levels 0d	No difference in the strength of young and old rats without injury at higher compliance in old rats; after injury young animals regained about 60% of the strength and compliance whereas old rats regain only 15% of its original force and modulus. At 3d wound is completely closed at in young rats; old rats have granulation tissue at 7d, no complete healing. Old rat had delayed and prolonged expression of MIF. Old rats showed lower levels of E2
Mattsom et al., 2005 [135]	Baboon; 1. Virgin young (5y), 2. Aged multiparous (23y)	N=6	Vagina	POP-Q	No clinical signs of POP

WB: western blot, d: day; m: month, y: year; OVX: ovariectomy, LOXL 1-4: Lysyl oxidase-like proteins 1-4, LAM: levator ani muscle; CMT-8: chemically modified tetracycline-8 (matrix metalloproteinase inhibitor); MIF: macrophage-migration inhibitory factor; POP: pelvic organ prolapse; POP-Q: pelvic organ prolapse quantification system; E2: estradiol, P4: progesterone;

Appendix 1.

ANIMAL MODEL FOR POP – SEARCH STRATEGY:

Note: search done 6.9.2019;

PUBMED:
Advanced, combine the 2 concepts with NOT (so delete the first NOT of concept 1)

EMBASE:
Advanced, switch off all embase mapping options, search 1st concept (delete the NOT in the beginning), search the second concept and then write #2 NOT #1

WOS:
Advanced, search just the core Collection.

Concept 1: animal model

PUBMED

NOT (human[Mesh] NOT animal[Mesh:NoExp])

EMBASE

NOT ('human'/exp NOT ('animal'/de OR 'coelenterate'/exp OR 'Mesozoa'/exp OR 'Placozoa'/exp OR 'sponge (Porifera)'/exp OR 'Deuterostomia'/de OR 'Ambulacraria'/exp OR 'Bilateria'/de OR 'Coelomata'/exp OR 'Protostomia'/exp OR 'Pseudocoelomata'/exp OR 'Chordata'/de OR 'Cephalochordata'/exp OR 'Hyperotreti'/exp OR 'Urochordata'/exp OR 'mammal'/de OR 'calf (mammal)'/exp OR 'monotreme'/exp OR 'amniote'/de OR 'reptile'/exp OR 'sauropsid'/exp OR 'tetrapod'/de OR 'Amphibia'/exp OR 'vertebrate'/de OR 'fish'/exp OR 'therian'/de OR 'marsupial'/exp OR 'placental mammal'/de OR 'Afrotheria'/exp OR 'Boreoeutheria'/exp OR 'Laurasiatheria'/exp OR 'Xenarthra'/exp OR 'Euarchontoglires'/de OR 'Dermoptera'/exp OR 'Glires'/exp OR 'Scandentia'/exp OR 'Haplorhini'/de OR 'tarsiiform'/exp OR 'primate'/de OR 'prosimian'/exp OR 'simian'/de OR 'Platyrrhini'/exp OR 'Catarrhini'/de OR 'Cercopithecidae'/exp OR 'ape'/de OR 'Hylobatidae'/exp OR 'hominid'/de OR 'orangutan'/exp OR 'Homo neanderthalensis'/exp OR 'gorilla'/exp OR 'chimpanzee'/exp OR 'nonhuman'/exp OR 'animal experiment'/exp OR 'model'/exp))

WOS
animal OR animal model OR mouse* OR mice* OR rodent* OR rat OR rats OR bovine OR rabbit* OR sheep OR ewe* OR primate* OR monkey* OR goat* OR hamster* OR swine* OR pig* OR porcine OR dog* OR beagle*

Concept 2: Pelvic organ prolapse

PUBMED
"Pelvic Organ Prolapse"[Mesh] OR pelvic-organ-prolapse*[tiab] OR urogenital-prolapse*[tiab] OR vaginal-vault-prolapse*[tiab] OR cystocele[tiab] OR cystocoele[tiab] OR "urinary bladder prolapse"[tiab] OR rectal-prolapse*[tiab] OR anus-prolapse*[tiab] OR uterine-prolapse*[tiab] OR vaginal-prolapse*[tiab] OR "genital prolapse"[tiab] OR "genito-urinary prolapse"[tiab] OR "genitourinary prolapse"[tiab] OR "pelvic descent"[tiab] OR "pelvic organ descent"[tiab] OR "pelvic prolapse"[tiab] OR "vaginal descensus"[tiab] OR "vaginal descent"[tiab] OR "vaginal wall prolapse"[tiab]

EMBASE
'pelvic organ prolapse'/exp OR 'pelvic organ prolapse*':ti,ab,kw OR 'urogenital prolapse*':ti,ab,kw OR 'vaginal vault prolapse*':ti,ab,kw OR cystocele:ti,ab,kw OR cystocoele:ti,ab,kw OR 'urinary bladder prolapse':ti,ab,kw OR 'rectal prolapse*':ti,ab,kw OR 'anus prolapse*':ti,ab,kw OR 'uterine prolapse*':ti,ab,kw OR 'vaginal prolapse*':ti,ab,kw OR 'genital prolapse*':ti,ab,kw OR 'genito urinary prolapse*':ti,ab,kw OR 'genitourinary prolapse*':ti,ab,kw OR 'pelvic descent':ti,ab,kw OR 'pelvic organ descent':ti,ab,kw OR 'pelvic prolapse*':ti,ab,kw OR 'vaginal descensus':ti,ab,kw OR 'vaginal descent':ti,ab,kw OR 'vaginal wall prolapse':ti,ab,kw

WOS
"pelvic organ prolapse" OR "urogenital prolapse" OR "vaginal vault prolapse*" OR cystocele OR cystocoele OR "urinary bladder prolapse" OR "rectal prolapse" OR "anus prolapse*" OR "uterine prolapse*" OR "vaginal prolapse*" OR "genital prolapse" OR "genito-urinary prolapse" OR "genitourinary prolapse" OR "pelvic descent" OR "pelvic organ descent" OR "pelvic prolapse" OR "vaginal descensus" OR "vaginal descent" OR "vaginal wall prolapse"

Chapter 3

The importance of developing relevant animal models to assess existing and new materials

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STRUCTURED ABSTRACT:

Purpose of review:

We summarize the recent literature on the use of different animal models for testing existing and new materials for treatment of pelvic organ prolapse.

Recent findings:

A wide spectrum of animal models is being used in urogynaecology, both for the study of physiologic and pathophysiologic processes, training in surgical procedures, yet mainly to study the host response to implant materials. The quality of studies is variable, and procedures, read-outs and reporting are not standardized. This makes comparison very difficult. The research community is experimenting with different knitting patterns, novel polymers, bioactivation as well as with resorbable rather than durable implants. Outcomes of the experiments are dependent on the location of implantation. Lighter polypropylene constructs seem to induce a less vigorous host response than elder heavier products. Modification of the surface yields contradictory findings. Resorbable acellular collagen matrices may be reintroduced, including as prophylactically inserted support structures.

Summary:

Though animal experimentation with novel candidate implants is advocated, there is a lack of standardization in reporting. The concept of resorbable construct is being revived, as durable materials have caused clinical graft related complications. Large animal experiments seem to provide interesting and more comprehensive information, yet their use may be contested.

Key words: mesh, polypropylene, polycaprolactone, polyurethane, tissue-engineering, pelvic organ prolapse

In vivo animal experimentation

Animal models are an essential part of medical research. They provide an opportunity to study (patho)physiological processes as well as testing therapeutic devices and drugs in a complex living organism (Table 1). The species used in translational experiments depends on the purpose of the study. In urogynaecology several models have been used to study pelvic floor changes throughout their lifespan as well as for treating pelvic floor dysfunctions (reviewed in [84]). In terms of studies on implants, experiments may have several purposes. They can be aimed at documenting the *host (inflammatory) response*, typically using histologic and molecular read outs representative for the wound healing process (reviewed in [66, 149]). Implants are inserted subcutaneously or at surgical sites that mimic the location anticipated for clinical use, such as the abdominal wall (as for hernia) or the vagina (as in pelvic organ prolapse). For the latter, larger species are more representative. For pelvic floor implantations, experiments may be dedicated to demonstrate the occurrence of local complications, such as exposure (also referred to as erosion[20]) or infection [150]. One can also study the mechanism of mesh contraction at the level of the pores or at the scale of the implant [151-153]. Other outcomes are the active and passive biomechanical properties of the explant. Biomechanical testing in this context is considered as a proxy of function. Active biomechanical testing measures contractile forces of the smooth muscle component of the vaginal wall. Conversely passive biomechanical testing measures stiffness, either uni-axial, bi-axial or by ball-burst testing. Loads can be either cyclical or single and/or until disruption. A wide range of variables can be reported [154]. Herein we provide background knowledge on the species used in translational experiments, followed by recent findings in such studies in the area of pelvic floor reconstruction and tissue regeneration.

Species used in urogynaecology

Rodents reach reproductive potential early on which ends already at 12-16 months (life expectancy three years). Typically inbred, genetically homogenous strains are used but transgenic animals are also available. In the context of implant research, their principal advantage is that their genome is annotated, and many molecular tests and immunostains are available. Limitations are their quadriped posture, small dimensions and the small fetal head-to-birth canal ratio. The supportive connective-tissue attachments of the vagina show many similarities to that of humans, both at the gross as well as microanatomy level [87]. The rat muscles best approximate the human pelvic floor muscles, followed by the mouse [155]. Also the basic anatomical and histologic structure of the vaginal wall is comparable [156]. Because of these similarities, the rat has been used to study (patho-) physiological changes in the pelvic floor and vaginal structures around crucial lifespan events (pregnancy, vaginal delivery, aging, menopause) [113, 157, 158] (reviewed in Callewaert [159]**) . They do not develop *spontaneously* prolapse but POP can be induced by knocking out specific genes related to elastin metabolism and fibulin-5 [160]. Rodents are however best known as models for reconstructive surgical procedures, either by covering (as in an incisional hernia) or reinforcing primarily sutured (as an overlaying mesh) full-thickness abdominal wall defects.

Rabbits are medium sized, live longer, and have an abdominal wall with a relatively large surface, making it possible to test multiple implants simultaneously or perform more comprehensive biomechanical testing. The elastance (change in intra-abdominal pressure per unit change of intra-abdominal volume) of the anterior abdominal wall in rabbits is rather high [161], therefore it seems a good and sensitive model for testing the biomechanical behavior of implants. The literature on experimental abdominal wall repair has been elegantly reviewed by Bellon [162] **. Rabbits are a higher species, more prone to infection and molecular tools are limited. Though their muscle mass is close to that of humans, the ultra-structure of their pelvic floor muscles differs widely [155]. *Vaginal* implantation has been shown possible, yet the complication rate is higher than clinically observed, and biomechanical testing is difficult because of the dimensions [163]. The rabbit vagina is also structurally very different: its cranial two thirds are actually the equivalent of the cervix. The wound healing process in the vagina is faster than that in the abdominal wall, yet slower in ovariectomized animals [164].

Pigs and *dogs* are rarely used, though their pelvic floor anatomy and tissue characteristics have some similarities to that of humans [165]. *Sheep* are increasingly used large animal models. Their fetal head-birth canal-ratio is close to that of humans and the pelvic floor anatomy has similarities to that of humans, despite being a tailed quadruped with different pelvic orientation [100] **. They can develop postpartum vaginal prolapse, which is of different nature than typical clinical POP. They can also develop rectal prolapse when pudendal and rectal innervation is compromised due to tail docking [166]. The effect of pregnancy, labor and delivery and menopause are documented by Urbankova [89] *. Because of vaginal dimensions comparable to humans, they are also used for training in or simulation of vaginal surgical procedures, including single incision surgery and implant insertion [167-170].

Closest to humans are **non-human primates**, which are in Europe virtually not used for (urogynecological) research because of ethical constraints. Mostly used are the rhesus macaque and squirrel monkeys. Both have anatomical and physiological similarities to humans [102, 171, 172]. Macaque pelvic support structures are similar to those of women. Their vaginal submucosa also attaches laterally to the levator ani muscle. The levator ani muscle originates along the obturator internus and connects to an analog of the arcus tendineus fasciae pelvis. The vaginal wall is mainly composed of collagen, elastic fibers, and smooth muscle, with nerves and blood vessels interspersed throughout. It is estrogen and progesterone sensitive, and the steroid receptors are in the basal epithelial, fibroblast, and smooth muscle cell nuclei [102]. Macaque and squirrel monkeys both *spontaneously* develop POP as a consequence of vaginal delivery, though in the latter species the proces is also age dependent.

Experiments with Ancillary Devices

Tsivian et al. (2016) compared the pullout strength of nickel and titanium alloy-anchoring devices or sacrospinous fixation in pigs to that in female cadavers, without showing a difference [173]. Of note to translational researchers is that this recent report mentions “relevant anatomical differences”, such as the lack of an arcus tendineus or sacro-uterine ligaments, but no reference can be found to this. The anatomy of the sacro-uterine ligament has however earlier been described in pigs [174].

Urbankova et al. (2017) used sheep to demonstrate the feasibility of performing trochar-assisted single incision vaginal mesh insertion into the rectovaginal septum. The implant was purposely designed and was pulled through the adductor magnus and external obturator muscles and the obturator foramen or sacrotuberous ligament and coccygeal muscles. This approach was later used to study the host response to flat and anchored textile meshes [175, 176].

Synthetic durable textile materials.

PP, as other durable materials, induces a foreign body reaction. The larger the contact surface, the more vigorous this response will be [47, 66]. Therefore, attempts were made to reduce the surface contact, e.g. by making more open and lighter constructs)[177], potentially by adding resorbable fibers, or covering the PP-fibers with substances that generate a lesser response. Several recent studies confirmed this.

Bigozzi et al. (2017) compared the biomechanical properties of subcutaneously inserted light- (16 g/m²) and heavy-weight (62 g/m²) polypropylene (PP) mesh in rats. Light weight explants provided comparable strength, at least when implanted in the transversal axis. When oriented along the longitudinal axis, the light weight explants were more compliant yet did bear a lesser load [178]. This shows, as earlier demonstrated, the relevance of anisotropy of materials [179].

Ge et al. (2016) soaked PP in dopamine to link a powder derived from small intestinal submucosa (SIS). Unfortunately, details about the nature of PP are missing. The combination indeed induced a less pro-inflammatory response, with an M2-dominated cell infiltrate and cytokine profile, when implanted in the vaginal submucosa of the rat [180].

Another modification tested is the coating of PP with basic fibroblast growth factor (bFGF) through polydopamine. This renders PP-meshes more hydrophilic, would encourage cell adhesion hence expedite wound healing. When used for reconstructing full thickness abdominal wall defects in rabbits, **Zhang et al. (2016)** [181] demonstrated less inflammation, a more organized collagen deposition, and an increased tensile strength.

The use of platelet-rich plasma (PRP) is being advocated for many indications, including mesh reconstructions. Platelets contain many growth and other bioactive factors, including Platelet-Derived Growth Factor, Transforming-Growth Factor, Insulin-like Growth Factor, believed to contribute to a balanced wound healing process [182]. Recent Cochrane reviews on chronic wound healing and on musculo-skeletal soft tissue injuries question the level of evidence currently available [183, 184]. **Avila et al. (2016)** compared the host response to subcutaneously inserted PP, with or without PRP, in the rabbit. PRP induced more inflammatory cells at 90 days, collagen I and III as well as total collagen after 7 days [185]. Later on the same group (**Parizzi et al.**, **2017)** did similar experiments by inserting a *macroporous* PP, again with or without PRP, in the rectovaginal septum without fixation. Vaginal exposure was observed in both groups. In the animals without exposures, there were no differences in the inflammatory infiltrate apart from more immature collagen [186]. The above experiments have partially conflicting results, neither were they designed to explore mechanisms behind these differences. Given no details on the exact PP implants were reported, it is difficult to comment on their relevance.

Ulrich et al. (2017 *) hypothesized that making hybrid PP partly resorbable by adding two thirds of polylactic acid (PLA) fibers would modify the biocompatibility. In this particular experiment a not further characterized PP implant was sandwiched between PLA fibers. In rats, this hybrid modified the host response minimally, i.e. it increased collagen 1 deposition after half a year. The biomechanical properties were not different, neither was the local complication rate [187].

Darzi et al. (2016) introduced novel methods to characterize the medium term host response to commercially available collagen coated PP implants, either by covering it with a cross-linked porcine acellular collagen matrix (ACM) or by coating the fibers with atelocollagen. In sheep, these implants modified the active and passive biomechanical properties. Addition of collagen did not reduce graft related complications [169]. Yet at the microscopic level, meshes containing collagen evoke different tissue responses at different times, yet both ultimately lead to physiological tissue formation approaching that of normal tissue [188].

In another experiment, the fate of flat Polyvinylidene Fluoride (=PVDF) transvaginal mesh was compared to that of a vaginal mesh with arms (**Urbankova et al. (2017) •**). Magnetic resonance imaging (MRI) was used as a non-invasive tool to quantify *in vivo* longitudinal geometrical changes in the mesh used for rectovaginal reconstruction. To visualize the mesh on MRI, Fe_2O_3 particles are mixed with the polymer in the production phase. This actually also facilitates visualization of the meshes on ultrasound [189]. The main finding was that the mesh flat surface area (32%) as well as the arms (17%) were significantly less within days after implantation, where after minimal changes take place [190]. This suggests that mesh contraction can be explained through a combination of pore aggregation, folding, plastic deformation, and actual contraction, as earlier demonstrated in rats and rabbits[152, 191]. The segmentation of MRI-visible meshes was therefore clinically applied to sacrocolpopexy [153]

The pioneering group of **Moalli** from Pittsburg **(2016)** used Rhesus monkeys to evaluate the impact of three PP meshes of different stiffness on vaginal smooth muscle and nerve structure and function. In this experiment two more recent lighter weight meshes were compared to an elder type (42 g/m²) and sham controls. Animals were middle aged yet reproductive, had no POP and underwent sacropexy after hysterectomy. They demonstrated that PP mesh has an overall negative impact on smooth muscle contractility and nerve density, the magnitude being a function of mesh properties. The larger the porosity, the lesser the impact on smooth muscle function, confirming the theory of stress shielding [192, 193] The experiment included an anisotropic hybrid implant

(containing resorbable polyglecaprone, for handling). It showed that mesh orientation has an effect, as earlier demonstrated in lower species [179].

Liang et al ** subsequently compared outcomes of coverage of PP (42 g/m²) with a non-cross linked acellular porcine urinary bladder matrix, either as a two or six ply layer (MatriStem Surgical Matrix RS, ACell Inc, Columbia, MD) using PP-only and sham controls [194, 195] **. Collagen coverage attenuated the host response to PP, i.e. the inflammatory response was less and vaginal contractility improved. More importantly, the host response to acellular porcine urinary bladder matrix *alone*, induced the most favorable response, in terms of biochemical, biomechanical and morphologic outcomes, comparable to sham operated animals [196]. The strategy of using an ACM only will therefore be discussed more in detail below.

Non-textile durable and resorbable synthetic meshes.

Several groups are experimenting with electrospun rather than textile matrices, which, in theory, have a structure close to the extracellular matrix, hence should promote tissue ingrowth. As in textiles, the polymers can be either resorbable or durable. The rationale of absorbable materials is that they do no persist eventually limiting in time local side effects. Under the assumption that resorbable scaffolds also have to provide physical strength, the replacing host tissue should be induced and remodeled fast and strong enough, to avoid mechanical failure. Several groups experiment with for that purpose “bioactivated” implants, to which certain factors that modulate the host response, are added.

Roman et al. (2016) tested electrospun *resorbable* Poly-L-lactic acid (PLA) and *durable* polyurethane (PU) meshes for abdominal wall reconstruction in rabbits. As a reference two synthetic *non-resorbable* textile products were used (PP and PVDF). As hypothesized, both electrospun meshes induced a M2-dominated response hence constructive remodeling, with more neovascularization, and extracellular matrix deposition, without a difference between the durable and resorbable electrospun meshes. Conversely textile implants generated a sustained (M1) chronic inflammatory response. There was no difference in compliance [197].

Several other electrospun meshes were tested of which some failed biomechanically. **Glintvad et al (2018)** and **Hympanova et al (2018)** spun meshes from resorbable *polycaprolactone*. Both observed the occurrence of herniation relatively early on, either in rats [198] or rabbits [198 199]. This limitation was not overcome by increasing the polymer load either by thicker meshes or larger fiber diameter [198] or by bioactivating it by basic fibroblast growth factor (bFGF) [199].

Electrospun resorbable (polycaprolactone) and non-resorbable (polyurethane) meshes were also tested in the rectovaginal septum of sheep, using light-weight macroporous PP as controls (18g/m²). In this model, failure cannot be tested, yet there were no graft related complications. Electrospun meshes induced a vigorous inflammatory infiltrate, which the authors tied to the large surface area in electrospun meshes. With the methods used there was no difference in host response between the two electrospun meshes [175].

Wu XJ et al. (2016) used co-electrospun poly[l-lactide-co-caprolactone] and fibrinogen, which may serve as a matrix for cell therapy applications. They compared these to polypropylene meshes for the reconstruction of a large abdominal wall defect in adult Beagle dogs (weight:15-20kg)[200]. This is a rather unusual model in Western countries, but these Chinese researchers suggest that dogs obviate the need for non-human primate research. There was no mechanical failure and the novel material induced earlier vascularization, a more organized connective tissue and apparently also muscle regeneration when compared to PP, on which further details were lacking [200].

Acellular matrices (ACM).

Bioscaffolds, derived from extracellular matrix (ECM) promote regeneration in different tissues, including skeletal muscle and tendons, by facilitating a site specific constructive remodeling response [201]. ACM may be of different origin (autologous, allografts and xenografts). ACM can be processed to modify their degradation pattern. Moalli revived the use of ACM *to (prophylactically) reconstruct the level-I (uterosacral ligaments) and II (paravaginal attachments) support apparatus*, following transection at the time of abdominal hysterectomy. They used porcine urinary bladder non-cross linked ACM (Matristem, 6-ply, which has a stiffness comparable to that of a 42 g/m² PP tape) to reconstruct the uterosacral ligaments and paravaginal attachments in Rhesus monkeys Outcome measures were biomechanical & biochemical properties and histology of the vagina over the ligament insertion site. Control tissues were vagina over a synthetic mesh support from another experiment as well as sham operated controls. After three months, the neo-ligaments were replaced by fibrous, vascular and fat tissue, providing at first glance adequate support. There were no differences in biochemical and biomechanical parameters, smooth muscle thickness and host response as compared to sham-operated controls, except for a 41% decrease in vaginal stiffness and a 25% increase in collagen III/I ratio in the group when the neo-ligaments were inserted vaginally rather than abdominally [195] **. The researchers explain this by a difference in tissue remodeling following vaginal or abdominal surgery. Even though these non-human primates are probably the closest model for clinical POP surgery, there are some limitations to these studies. The primates were middle aged, in their reproductive stage, without cycle synchronization, and without prior prolapse [195, 196]. It remains at present difficult to extrapolate those findings to surgical practice, but this among the best research in the field.

Cell based solutions.

Another approach is a tissue-engineering strategy where one adds cells to reconstructive matrices[202]. In two experiments two different matrices loaded with two different xenogenic cell types [202, 203] were inserted subcutaneously in immune-modulated mice. **Darzi et al. (2018) ••** documented the macrophage phenotypes induced by polyamide knitted mesh (42 g/m²) coated with gelatin, with or without Endometrial Mesenchymal Stem/Stromal Cells (eMSC). They ran experiments on immuno-deficient (Nod-Scid-IL2Rgamma^{null}) and immuno-competent mice (C57BL6). The addition of eMSC modified the macrophage phenotype from M1 to M2 in both mice models. Survival of grafted cells was limited in immunocompetent mice, whereas in the immune-deficient group they were demonstrable up to 7 days [204].

Wu QK et al. (2016)• seeded human adipose-derived stem cells (hASCs) on acellular bovine pericardium (Shanghai Cingular Biotech Corporation, China) that was implanted subcutaneously in athymic nude mice for up to 12 weeks. Seeding was done either during the surgery (“in vivo”) or by three days culturing “in vitro” before the surgery. Bioluminescence suggested persistence of hASCs for three months, with more cells in the in vivo group with minimal spread elsewhere. Histology at three months revealed that in vivo seeding induced more cell ingrowth, neovascularization and collagen content [202]. These experiments now logically should be translated to a larger animal model and a vaginal environment. This may be rather difficult as they require immunomodulation. Also outcome measures should be more specific to PFD.

Key points:

- There is not one animal model that fits all purposes.
- The lack of standardized implantation and reporting methods make comparison of experiments difficult.
- The relationship between morphology, biomechanical testing and function remains unclear.
- Several light-weight polypropylene implants yield low local complication rates.
- The use of “visible” implants permits studying implant deformation longitudinally.

- A novel strategy may be the preventive use of acellular collagen matrices to suspend pelvic floor structures.

Conclusion:

Novel material should be tested in representative animal models, in order to predict local complications and the host response one can expect. The research community is experimenting with different knitting patterns, novel polymers, and bioactivation as well as a strategy of using resorbable implants. Outcomes are dependent on the location of implantation. Lighter polypropylene constructs seem to induce a less vigorous host response than elder products. Modification of the surface yielded contradictory findings. Resorbable acellular collagen matrices may be reintroduced, including as prophylactically inserted support structures.

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Table 1: Anticipated time line for the introduction of novel devices into the market as proposed by the International Urogynaecological Association.[24]

Steps	Goals	Time line
Pre-marketing, nonclinical		
1. Preclinical file	Accurate description of product - toxicity studies for new polymers	0–6 months
2. Preclinical testing - animal	Host inflammatory response	0–12 months
3. Cadaveric studies	Anatomical documentation	6–12 months
Pre-marketing, clinical		
4. Clinical studies: phase II trial	Efficacy study	12–24 months
	Long-term safety	Ongoing
5. Clinical studies:		
temporary registry	Surveillance study (n=1 000)	30–42 months
“Yellow card” - MAUDE reporting?	Self-reporting on a larger scale	Ongoing
Recommended: RCT	Should prove whether product/procedure is advantageous/competitive	Should be conceived as early as possible

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Chapter 4

Effects of non-ablative Er:YAG laser on the skin and the vaginal wall: Systematic review of the clinical and experimental literature

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ABSTRACT:

Introduction and Hypothesis: Er:YAG laser is frequently used in dermatology and gynecology. Clinical studies document high satisfaction rates, however hard data on the effects at the structural and molecular level are limited. The aim of this systematic review was to summarize current knowledge about objective effects of non-ablative Er:YAG laser on the skin and vaginal wall.

Methods: We searched MEDLINE, Embase, Cochrane and the Web of Science. Included were studies investigating objectively measured effects of non-ablative Er:YAG laser on the skin or vaginal wall. Included were studies of any designs. Due to the lack of methodological uniformity, no meta-analysis could be performed and therefore results are presented as a narrative review.

Results: We identified *in vitro* or *ex vivo* studies on human cells or tissues, studies in rats and clinical studies. Most studies were on the skin (n=11); the rest were on the vagina (n=4). The quality of studies is limited and setting of the laser was very diverse. Though the methods used were not comparable, there were demonstrable effects in all studies. Immediately after application the increase of superficial temperature, partial preservation of epithelium and subepithelial extracellular matrix coagulation were documented. Later, an increase of epithelial thickness, inflammatory response, fibroblast proliferation, an increase of collagen amount and vascularization were described.

Conclusions: Er:YAG laser energy may induce changes in the deeper skin or vaginal wall, without causing unwanted epithelial ablation. Laser energy initiates a process of cell activation, production of extracellular matrix and tissue remodeling.

Key words: collagen, dermis, epidermis, histology, laser, resurfacing

Brief summary:

Er:YAG laser energy may induce changes in the deeper skin or vaginal wall, without causing unwanted epithelial ablation.

INTRODUCTION

Lasers are devices that emit electromagnetic radiation of a specific wavelength. The effects on the tissue are due to conversion of the absorbed energy to mechanical and thermal energy. Ablation refers to the mechanical disruption of tissue due to delivery of energy that is sufficient to produce thermal explosions or shock waves within the tissue leading to its disintegration [1]. Thermal effects include denaturation of proteins, coagulation and necrosis of tissue [2]. Based on those effects, lasers can be divided in to ablative or non-ablative (thermal).

Historically, lasers have been often used in dermatology. Based on active medium, the most frequently used lasers in dermatology are CO_2 (wavelength: 10,600nm) and Er:YAG (2940nm). This review focus on non-ablative Er:YAG laser, which cause less complications compare to ablative CO_2 [3]. In both instances, the laser performs a controlled tissue injury, which induces a healing response, which may lead to the desired structural and functional improvement of the tissue. “Laser resurfacing” is used to treat wrinkles, scars or dyspigmentation [4]. Morphologically, the aged skin is atrophic, epidermis is thin, dermis has reduced cell turnover and number of fibroblast, the collagen bundles are less in number and thin [5].

Recently, lasers are increasingly being used in gynecology and pelvic floor medicine, including for genitourinary syndrome of the menopause (GSM), stress urinary incontinence (SUI), pelvic organ prolapse (POP) or vaginal relaxation syndrome [6]. In the case of GSM, caused by a combination of aging and estrogen depletion, the vaginal wall is atrophic. Morphologically represented by generalized thinning of the epithelium [7], lamina propria and muscularis [8] and reduced epithelial glycogen content. The collagen undergoes hyalinization, the elastin becomes fragmented [9–11] and vascularisation diminishes [8]. Also in SUI and POP, extracellular matrix changes have been described, yet the exact nature of these remain controversial [12–16].

In all above mentioned conditions, the goal of laser treatment is the restoration of the structure and function of the skin or vaginal wall. Clinical studies report high satisfaction rates in both fields, yet objective documentation of the laser energy impact at histological and molecular level is more documented on skin [17,18].

MATERIALS AND METHODS

Objective

To define the current knowledge on the effects of “non-ablative” Er:YAG laser on the skin and on the vaginal wall. The primary outcome is to quantify histological effect of Er:YAG laser on the vaginal wall and whether this effect can be seen in the connective tissue without epithelial ablation. Secondarily, we aim to examine if changes found in the vaginal wall were previously described on the skin. In both outcomes, we search for quantifiable evidence underlying the clinical laser effects.

Protocol and registration

This systematic review was structured in line with the guidance provided in the Preferred Reporting Items for Systematic reviews and Meta-analyses (PRISMA) statement [19]. The research question was: “What is the current knowledge about the effects of ,,non-ablative,, Er:YAG laser on the skin and the vaginal wall?” This review was registered, and its protocol has been previously published at the National Institute for Health Research PROSPERO, International Prospective Register of Systematic Reviews (http://www.crd.york.ac.uk/PROSPERO, registration number: CRD42018085763).

Information sources, search strategy

A complete computerized literature search was conducted using MEDLINE (Pubmed), Embase, Cochrane and the Web of Science including all studies without date and language restriction up to 7th January 2018. The electronic search strategy included both Medical Subject Headings (MeSH) and keywords (Appendix 1). Endnote X8.2 (Clarivate Analytics, Philadelphia, Pennsylvania, United States) and manual search were used to eliminate duplicate reports. Duplicates were divided into type-I (duplicates among different databases) and type-II (duplicate publications in different journals/issues) duplicates. Reference lists of original articles and topic-related reviews were checked manually to identify further relevant articles.

Eligibility (Studies selection, inclusion and exclusion criteria)

Two reviewers (LH and KM) independently screened titles and abstracts for eligible articles. Any disagreement involving the eligibility was resolved through discussion. Studies investigating objectively measured effects of non-ablative Er:YAG laser (2940nm) on the skin or vaginal wall were included. The following eligibility criteria were entailed: Study design: all study designs; Participants: humans of any gender or experimental animals, ex vivo tissues, in vitro tissue models; Intervention: Er:YAG laser application; objective outcomes: histology, immunohistochemistry, electron microscopy, vascularisation measurements, imaging, biochemical analysis, molecular analysis or other measurable outcomes; articles published in English. Studies reporting only subjective outcomes, assessed as either by the patient or the care provider, were not included. Studies performed on cell cultures without tissue structure were excluded. Review articles, commentaries, letters to the Editor and unpublished articles (i.e. conference abstracts) were excluded.

Screening Methods and Data Extraction

Full text of articles, meeting the criteria by title and abstract, were screened and checked for agreement. The information from these studies was tabulated according to the outcome (histology vs. others) and the nature of the subjects (human, animal, ex vivo studies).

Statistical analysis and risk of bias assessment

The obtained data are reported as a narrative review. Study quality and risk of bias were assessed using ROBINS-I: a tool for assessing the risk of bias in non-randomised studies of interventions [20].

RESULTS

Literature search

Initially, 7187 publications were identified by the search. There were 2019 duplicates eliminated for various reasons as displayed in Figure 1. The abstracts of 5168 articles were screened for inclusion and exclusion criteria, and again most were excluded (n= 3895) because they were on findings with other laser types, most frequently Neodymium:YAG. Eventually, 341 full-text articles were assessed but another 326 articles were excluded because they were review articles, using laser in the ablative mode or using histology for other purposes (i.e. diagnosis of the underlying disease or completeness of the resection) or the laser was used to enhance drug delivery. One excluded study did not clearly specify whether laser was ablative or not [21], and two studies used Er:YAG laser to deliver non-ablative acoustic waves, yet not for its thermal effects [22,23]. Reviews were screened for potential original articles however not even one could be included in the systematic review.

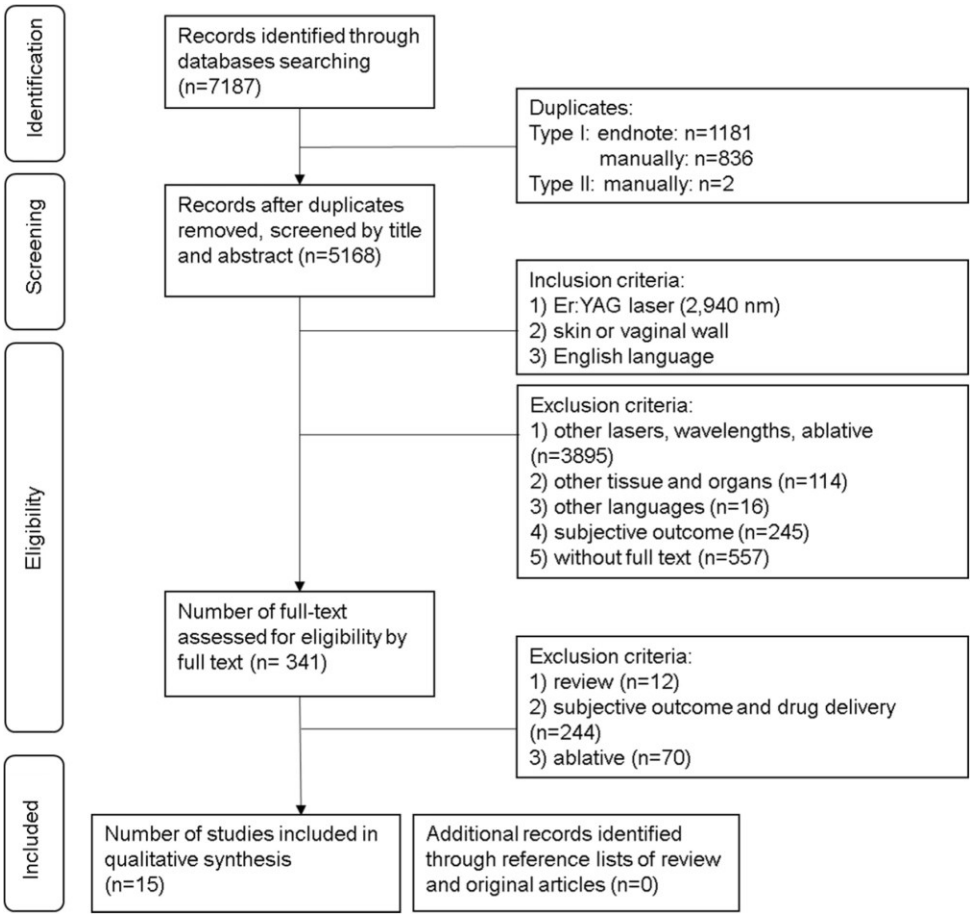

Figure 1: Systematic review of the effects of Er:YAG laser on the skin and vaginal wall. PRISMA flow diagram for studies retrieved through the searching and selection process.

Statistical analysis and risk of bias assessment

Due to the lack of methodological uniformity of the included studies, no meta-analysis could be performed and therefore we present the results as a narrative review. Assessment of risk of bias is reported in table 1

Effects of non-ablative Er:YAG on the skin and vaginal wall

All included studies (Table 2) were prospective, 12 were controlled, and none were randomized controlled trials. The settings of the laser in these studies are displayed in Table 3, and were very diverse. We identified three studies reporting laser effects *in vitro* or *ex vivo* [24–26] on human cells or tissues. There were two studies in rats [27,28] and 12 human studies. Most studies were involving laser application on the skin (n=11); the rest were on the vagina (n=4).

Though the methods used were not comparable, there were demonstrable effects in all studies. Several readouts were used (Table 4), including histology (n=13) using H&E stains for basic structural assessment, Alcian blue for collagen, Van Gieson for elastin [29], PAS-reaction for glycogen, and picrosirius red for newly formed collagen. Immunohistochemistry was used for epidermal injury (keratin-16), cell proliferation and repair (Ki-67, p53), the integrity of the basal membrane (laminin, collagen IV, VII), macrophage marker (CD68). Myofibroblasts and their activation were demonstrated by alpha-smooth muscle actin and procollagen-I. Cytokine expression was demonstrated for TGF-β, IL-1β and IL-8.

Bellow, we report the effects on the skin and vagina in two separate paragraphs. First paragraph includes separately reported data obtained from tissue model and animal studies. Further we describe only human studies, first the effects on the epidermis/epithelium, and then the dermis/lamina propria. We did not find studies that focused on the impact of Er:YAG laser on the lamina muscularis.

In brief, the effects of laser can be summarized as follows. Five studies demonstrated partial [24,27,28,30,31] preservation of the epithelium immediately after laser application. In all studies was a demonstrable effect on the epithelium, with partial or full loss of integrity at the time point studied. There were demonstrable other acute effects, such as a superficially increased temperature [25,26,32] and collagen coagulation [27,28,30] in the subepithelial layers. More delayed effects were demonstrated in the epithelium (an increase of epithelial thickness and the presence of glycogen) and the subepithelial layer (increased number of fibroblasts, increased amount of collagen and increased degree of vascularization).

1/ Effects on the skin

Tissue culture model and animal studies

Studies investigating the effects of Er:YAG laser on the skin were performed *ex vivo* on tissue-engineered skin (n=1) or *in vivo* in rats (n=2). Immediately after application, on tissue-engineered skin as well as on rat skin, sub-ablative Er:YAG application induced partial epidermal ablation [24,27,28]. The coagulation was demonstrated *in vivo* in a rat model, at 300µm below the epidermal-dermal junction [27,28]. One week after application in a skin-tissue-culture an increase in the number of fibroblasts was demonstrated as compared to non-lasered control [24].

Human studies

Studies investigating the effects of Er:YAG laser on the human skin were performed *ex vivo* on explants (n=2) or i*n vivo* (n=7).

Immediate effects in the epidermis

Two studies measured *ex vivo* the temperature on human skin explants immediately after the laser application. One protocol demonstrated a maximum temperature of 60°C up to 10µm below the surface measured with a thermal camera [25]. Another protocol reached 70-80°C at the surface measured with a digital probe [26].

In vivo, the laser effect ranged from preservation of the basal keratinocytes to ablation with preservation of the basal membrane only or complete epidermal ablation [30].

Delayed effects in the epidermis

The delayed thermal effects were documented between 24 hours untill month(s) after the initial laser application. There was no homogeneity in the methods used to assess the impact of laser. Twenty-four hours after laser application there was minimal epidermal damage, detected by the presence of keratin 16 positivity, loosening of the stratum corneum and epidermal cell cohesion. Laminin γ2 confirmed the integrity of the basement membrane and increased proliferation of keratinocytes evidenced by Ki67 expression. Matrix metalloproteinase (MMP) regulators increased (cJun, JunB) as well as the MMPs themselves; transcription levels of MMP-1 (230-fold), MMP-3 (140-fold) and MMP-9 (10.4-fold) rose above baseline ($p<0.05$). MMP-1 and MMP-3 gene expression, as well as proteins, were located in both epidermis and dermis [31]. Epidermal damage or partial ablation (vacuolar cytoplasmatic degeneration, eosinophilic clamped keratinocytes) resolved 3 to 7 days after treatment [30,31]. Keratin 16 was substantially elevated for 2 weeks [31]. Level of heat shock protein 70, a sensitive indicator of the skin's acute response to heat was not altered [31]. Ki67 increased 5-fold vs. baseline by 3 days after laser treatment ($p<0.05$), and returned to baseline at day 7 [31].

Two weeks after treatment an increase in epithelial thickness was observed (mainly the stratum spinosum), however not quantified [26]. Later on the increase in epithelial thickness was measured (before: 54.3 ± 6.3µm, after 3 months of treatment: 63 ± 6.5 µm ($p=0.042$), 3 months after last treatment i.e. 6 months from the start: 66.4 ± 5.1µm ($p=0.005$ to baseline) [33]. Those changes were not accompanied by a change in p53 immunostaining [34]. The p53 is a marker of cell stress, indicates an arrest in cell proliferation and DNA repair or may indicate impending programmed cell death [35]. The p53 expression was comparable to what was measured before treatment as well as in non-lasered controls [34].

Immediate changes in the dermis

Change of temperature, impact on fibroblasts and extracellular matrix were investigated. A temperature of 32°C was measured *in vivo* in one volunteer where a probe was placed at 335µm under the skin surface under the optical coherence tomography (OCT) control [26]. Thermal collagen coagulation visualized as hyalinization and glass-like appearance is a principal marker to determine the depth of thermal impact. The coagulation was demonstrated *in vivo* 120µm below the epidermal-dermal junction [30].

Delayed effects on the dermis.

Later effects on the dermis included observations ranging from days to months after laser application. The impact on the inflammatory response, fibroblasts, extracellular matrix, and vascularization was investigated. Within the first

day, there was an increase in inflammatory cytokines interleukin IL1β (16-fold vs baseline, p<0.05) and IL8 (200-fold vs baseline, p<0.05), a potent chemoattractant for polymorphonuclear leukocytes. The tumor necrosis factor alfa (TNF-α) was not significantly upregulated. A substantial cellular infiltrate of polymorphonuclear cells was detected, frequently elastase positive neutrophils. Neutrophil infiltration extended from the dermis into the epidermis and declined between day 3 and day 7 [31]. One week after application infiltration by CD68 positive macrophage/monocyte and virtually no neutrophils were documented in another study [30]. Two weeks after application, the infiltrate was still present, but mainly around the vessels, as evaluated by histology and OCT [26].

Three weeks post-application higher counts of stellate myofibroblasts (that have abundant eosinophilic cytoplasm and large nuclei, and that stain for alpha-SMA) were present down to 120µm in vivo [30]. Activated fibroblasts expressing pro-collagen immunostaining were found four weeks after application at a depth of about 320µm (untreated 5.6% vs. after treatment 25.5% positive cells) [26].

The TGF-β (a major regulator of the synthesis of the extracellular matrix) increased after treatment (before: 10.1 ± 1.4% of positively stained area, at the end of treatment 15.9 ± 2.3, three months after: 12.6 ± 2.9; p <0.05). Expression of TGF-β at the end of therapy was comparable to that of control unexposed subjects below 30 years of age, and significantly higher compared to older unexposed controls (over 30 years) [36]. One week after application, there were remnants of degenerated collagen in the zone of regeneration [30]. One and two weeks following Er:YAG laser, levels of procollagen I and III mRNA and procollagen I protein were significantly elevated (p<0.05). Immunohistochemistry revealed that Er:YAG laser treatment stimulated procollagen I synthesis in fibroblasts throughout the mid to upper dermis [31]. Four weeks post-treatment thin collagen bundles were visualized in the upper dermis as more tightly packed with a parallel orientation to the skin surface [26]. At the end of 3 months treatment, collagen types I, III, and VII, as well as newly synthesized collagen (Picrosirius red) and tropoelastin increased, while the mean level of total elastin was significantly decreased [33,36].

2/ Effects on the vaginal wall - Human studies

Effects on vaginal wall were described only in women. In other words, there were no *ex vivo*, animal, or *in vitro* tissue studies. Three studies focused on the treatment of SUI [37–39], one on GSM [40].
Immediate effects on the epithelium

A temperature of 45-65°C was measured with a thermal camera at a depth of 50µm, using different delivery handpieces and a non-ablative Er:YAG laser setting [32]. No studies focused on the epithelial structure immediately after application.

Delayed effects on the epithelium

Two months after laser treatment, in women with SUI, the epithelium became thicker by 64.5%, the glycogen content of the cytoplasm increased and other degenerative signs in the epithelium became reduced [38]. The latter included cellular disintegration, perinuclear vacuoles, a looser and thicker shedding surface in the epithelial layers, gaps between the cells, impaired epithelial basal relief, and a thickened basal membrane. Epithelial proliferative activity (Ki-67) increased from 19.05±2.86% to 31.79±2.25% (p<0.05) [37]. In women with GSM, 1-12 months after laser application, epithelial parakeratosis and acanthosis (increased epithelial thickness) were described however it was not quantified [40].
Lamina propria

No studies investigated immediate changes in lamina propria. In women with SUI, two months after laser application, enlarged stromal papillae and an increased count of fibroblasts with signs of synthetic activity were demonstrated [37]. The number of elastic fibers also increased and the fibers were long and perpendicular. Fine collagen bundles had a more orderly distribution [38]. Both the number of blood capillaries and density increased by 42.3% and 61.1%, respectively [37,38]. In women with GSM, the number of fibroblasts and fibrillar extracellular matrix components increased, there was vasodilation and pericapillary edema, major congestion, as well as a general improvement in vascularization was described, yet this was not defined or quantified [40]. In those women, the maturation index increased after application and the improvement persisted up to 12 months post-treatment (baseline: 20.8 ± 5.4, 12 months: 52.2 ± 8.5, $p<0.001$). This was accompanied by a decrease in vaginal pH (before: 5.0±0.4 vs. 12 months post-treatment: 4.4±0.6; $p<0.001$) [40]

DISCUSSION

The aim of this systematic review was to summarize the objective demonstrable effects of non-ablative Er:YAG laser on the skin and vaginal wall. Immediately after application an increase of temperature was demonstrated, usually with partial preservation of the epithelium, and demonstrable subepithelial extracellular matrix coagulation. Later in time, an increase in epithelial thickness, fibroblast proliferation, amount of collagen and vascularization was observed. However, the number of studies, as well as the number of evaluated subjects, are limited and there was no standardized methodology, making comparisons very difficult.

The main goal of non-ablative Er:YAG thermal mode is to cause remodeling of sub-epithelial layer. The best studied physiological remodeling *in vivo* is that of post-traumatic wound healing. Wound healing has been traditionally described as occurring in three phases: an inflammatory phase, a proliferation phase, and a remodelling phase [41]. The principal cell in the inflammatory phase is the macrophage (CD68), which is responsible for the degradation of the damaged tissue (wound debridement) and stimulates the influx and proliferation of fibroblasts by secretion of cytokines (IL-1, TGF-β) [42,43]. The presence of macrophages, an increase in IL-1 and TGF-β following laser application were demonstrated in reviewed studies [31,36]. Myofibroblasts play an important role in the proliferative phase after tissue injury. They participate in the active production of extracellular matrix components including collagen I and III [44]. Activated myofibroblasts with signs of synthetic activity were documented in three reviewed studies [26,30,31]. The third phase of wound healing is characterized by remodeling of the granulation tissue. In this stage, collagen III is replaced by collagen I and proteoglycans are synthesized. One study demonstrated an increase of both collagen I and III, however, did not use its ratio to confirm the remodeling process [33]. Those three phases of wound healing seem to be present in the healing process after laser application.

We have to point out that the chromophore (absorption target), of Er:YAG laser energy is water [45]. The percentage of water content is dependable on the type of tissues [46,47]. Therefore, preclinical testing and adjusted laser setting need to be set up prior usage at new tissue locations. Impact on tissue water content differ with the age [48] and menopause [49]. Those conditions should be also considered during personalized application. In the case of vaginal application specifically, pre-menopausal state or hormonal treatment may have an impact on treatment outcomes. Other specific differences between skin and vaginal wall are pilosebaceous hair follicles and sweat glands, which are present in the skin and participate on rapid healing and regeneration after the laser. While searching the literature, additionally to the skin and vagina we found other applications of non-ablative Er:YAG laser in other locations, such as the soft palate and gingiva. In the case of soft palate lasering, the clinically treated condition is snoring. In this location a controlled study on rats was performed [50]. A submucosal thermal effect was noted at 0.4 – 1.7mm depth 24h post-laser. Most of the epithelial tissue was preserved, there was a moderate grade of inflammation (score 2 out of 3). The inflammation remained at the same level of intensity for the first week, decreasing by the third week, to

completely disappear by week five. Those findings are in agreement with those reported in the dermis [26,30,31]. In dentistry, Er:YAG is used to treat i.e. periodontitis. *In vitro* Er:YAG promoted gingival fibroblast proliferation [51] comparably to fibroblasts retrieved from the skin [24] and induced up- and down-regulation of several proteins associated with wound healing. Up-regulated was i.e. galectin-7, which modulates fibroblast proliferation [52].

The outcomes used to evaluate effects were mainly histology and immunohistochemistry. In a few studies the temperature was measured [25,26,32], molecular analysis [31] or OCT [26] was done, or the maturation index or pH measurement was made [40]. The most frequently used histological stain was H&E. H&E is a basic stain which visualizes partial or full preservation of the epithelium, coagulation at the sub-epithelial layer and identifies an inflammatory response. Extracellular matrix changes were basically evaluated with relatively simple stains (Alcian blue, Van Gieson). Additional immunohistochemistry gave a wider insight into the type of inflammatory cells, or proliferative and synthetic activity of fibroblasts, types of collagens and vascularization. These methods are that easy that they should be included in future studies, so that basic comparisons between studies remains possible. Additionally, biochemical analysis as well as electron microscopy could extend our understanding of biochemical and structural changes of the extracellular matrix. The vascular changes may be properly quantified in future studies [53]. Temperature measurement was used in only a few studies, yet typically not in deeper locations (dermis, lamina propria). This would certainly be beneficial to understand the effects that may occur in the deeper layers. OCT and other ultrasound-based methods could be used as they are non-invasive and allow longitudinal observations. Also, mechanical properties before and after treatment might give insight into functional changes of tissue. In the case of the vaginal wall, the maturation index and pH were successfully used as a relevant outcome in the case of GSM.

Heat injury is the claimed mechanism of non-ablative Er:YAG to induce an effect. However, other mechanisms may play a role in regeneration and remodelling including vascular damage, recruitment of inflammatory cells and release of mediators [54]. The exact contribution of these mechanisms to the laser effect may be investigated. As disclosed by this review, many studies lack a comprehensive and structured strategy to quantify tissue changes, either when novel target tissues are considered, or to define appropriate laser settings for given indications. The use of *ex vivo* tissue models may be further considered and upgraded [24].

In summary, animal and clinical studies demonstrated that Er:YAG laser energy may induce changes in the deeper skin or vaginal wall, without causing unwanted epidermal ablation. Laser energy is transformed initiating a process of cell activation, production of extracellular matrix and tissue remodeling. However, the number of studies is limited and there is no standardization of measurement methods. Therefore we aim to conduct a preclinical study to investigate further the impacts of Er:YAG laser on the vaginal wall. We plan a study in a large animal model, being an ovariectomized sheep, to collect a set of data, that is beyond the level of the presented studies, and provides insight in read-outs that are difficult to collect in human beings.

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Tables:

Table 1: ROBINS-I: a tool for assessing risk of bias in non-randomised studies of interventions.

ROBINS-I: a tool for assessing risk of bias in non-randomised studies of interventions

	Pre-intervention		At intervention	Post-intervention				ROBINS I overall
Authors	confounding	selection	classification of interventions	deviations from interventions	missing data	measurement of outcomes	selection of the reported result	risk of bias
Tissue culture model								
Kao et al., 2003	low	low	low	low	low	serious	low	serious
Animal (rat)								
Majaron et al., 2000	low	low	low	low	low	low	low	low
Majaron et al., 2001	low	low	low	low	low	low	low	low
Human								
Orringer et al., 2011	low	low	low	low	low	serious	low	serious
Drnovsek-Olup et al. 2004	low	low	low	low	low	low	low	low
Kunzi-Rapp et al., 2006	low	low	low	low	low	serious	low	serious
El-Domyati et al., 2012	low	low	low	low	low	serious	low	serious
El-Domyati et al., 2013	low	low	low	low	low	serious	low	serious
El-Domyati et al., 2015	low	low	low	low	low	serious	low	serious
Gungor et al., 2014	low	low	low	low	serious	serious	low	serious
Lapii et al., 2017a	low	low	low	low	low	serious	low	serious
Lapii et al., 2017b	low	low	low	low	low	serious	low	serious
Gaspar et al., 2017	low	low	low	low	serious	serious	low	serious
Fistonic et al., 2016	no information	no information	low	no information	moderate	serious	moderate	serious
Lukac et al., 2010	low	low	low	low	low	serious	low	serious

Table 2: Summary of studies investigating the effects of non-ablative Er:YAG laser on vaginal wall and skin (A/ Histology and B/ Other outcomes, C and D summarise main results). Studies includes ordinal staining as well as immunohistochemistry (IHC). Used abbreviations: y – year(s), H&E – hematoxylin and eosin, SUI – stress urinary incontinence, PAS – periodic acid Schiff, OCT- Optical coherence tomography, MI – maturation index.

A/ Histology

Authors	Subject	Organ	Outcome measure (staining)	Time point prior treatment or control	Sessions count (interval between sessions)	Time points after last application
Kao et al., 2003	human tissue culture model	skin	H&E	Yes	one	day 0, 1 week
Majaron et al., 2000	rat	skin	H&E	Yes	one	1 hour
Majaron et al., 2001	rat	skin	H&E	Yes	one	1 hour, 1 day, 5 days, 4 weeks
Orringer et al., 2011	10 humans (44 -75y)	skin	IHC: laminin γ2, keratin 16, Ki67	Yes	one	1 day, 3 days, 7 days, and 14 days
Drnovsek-Olup et al., 2004	6 women (52-63y)	skin	H&E; IHC:Ki-67, CD68, α-SMA, collagen type IV	No	one	day 0, 7days and 21days
Kunzi-Rapp et al., 2006	7 humans (not specified)	skin	H&E, Alcian blue IHC: Human pro-collagen Type I	Yes	two (2 months)	2 weeks, 4 weeks
El-Domyati et al., 2012	6 women (38-72y)	skin	H&E, Verhoef-van Gieson, picrosirious red IHC: collagen types I, III and VII, elastin and tropoelastin	Yes	six (2 weeks)	end of treatment, 3 months

El-Domyati et al., 2013	6 women (38–72y)	skin	IHC: p53	Yes	six (2 weeks)	end of treatment, 3 months
El-Domyati et al., 2015	6 humans (37–72 y)	skin	picrosirius red IHC: TGF-β	Yes	six (2 weeks)	end of treatment, 3 months
Gungor et al., 2014	5 women (20-40 y)	skin	H&E, Van Gieson, Orcein, Masson's Trichrome	Yes	three (month)	4 weeks
Lapii et al., 2017a	18 women (37-62y) SUI	vagina	H&E, Pearl's Van Gieson, Weigert's resorcin-fuchsin, PAS	Yes	two (1-1.5 month)	1.5-2 months
Lapii et al., 2017b	18 women (37-62y) SUI	vagina	H&E, Pearl's Van Gieson, Weigert's resorcin-fuchsin, PAS, Ki-67	Yes	two (1-1.5 month)	1.5-2 months
Gaspar et al., 2017	6 women (post-menopausal)	vagina	H&E	Yes	three (3 weeks)	1, 3, 6, 12 months

B/ Other outcomes

Authors	Subject	Organ	Outcome measure	Time point prior treatment or control	Sessions count (interval between sessions)	Time points after last application
Orringer et al., 2011	10 humans (44 -75 years)	skin	IL-1β, IL-8, cJun and JunB proteins (AP-1 complex), (MMP-1), MMP-3, MMP-9, type I and type III procollagen	Yes	one	1 day, 3 days, 7 days, and 14 days
Kunzi-Rapp et al. 2006	2 humans (not specified)	skin	Temperature (ex vivo, in vivo) OCT	No (temperature)	one	temperature: immediately

				Yes (OCT)		OCT: 4 days, 14 days, 28 days
Lukac et al., 2010	human (ex vivo)	skin	Temperature	No	one	immediately
Fistonic et al., 2016	women SUI	vagina	Temperature	No	one	immediately
Gaspar et al., 2017	25 women (post-menopausal)	vagina	Maturation index, pH	Yes	three (month)	MI: 1 month, 3 months, 6 months, 12 months pH: 3 months, 12 months

C/ Histology – main results

Authors	Results
Tissue culture model	
Kao et al., 2003	the average fibroblast count (number per viewing field) increase in the lasered group as compared to control, 92.2 versus 48.5 ($p<0.01$)
Animal (rat)	
Majaron et al., 2000	collagen coagulation up to 280 µm depth while preserving the epidermis in a lasered group compare to no coagulation in control
Majaron et al., 2001	A/ collagen coagulation up to 270 µm depth while preserving the epidermis in a lasered group compare to no coagulation in control B/ depth of neo-collagen zone (tightly packed parallel collagen fibrils and an increased number of fibroblasts) up to 250 µm
Human	
Orringer et al., 2011	keratin 16 30-45% area stained versus 0% in baseline Ki67 elevated 5-fold versus baseline ($p<0.05$)
Drnovsek-Olup et al., 2004	depth of dermal injury 80 ± 12 µm
Kunzi-Rapp et al., 2006	pro-collagen expression laser 22.5% versus control 5.6% positive cells
El-Domyati et al., 2012	epithelial thickness increased 54.3 ± 6.3µm versus 66.4 ± 5.1µm after laser ($p=0.005$) collagen type I increased 64.0 ± 4.5% versus 71.1 ± 3.9% after laser ($p=0.04$);

	collagen type III increased 59.7 ± 2.1% versus 62.9 ± 1.1% after laser (p=0.037)
	newly synthesized collagen 11.3 ± 2.1% versus 16.1 ± 3.8% after laser (p=0.001)
	collagen type VII increased 8.4 ± 1.4% versus 11.7 ± 1.5% after laser (p=0.004)
	total elastin decreased 68.2 ± 7.8% versus 56.5 ± 4.5% after laser (p=0.03)
El-Domyati et al., 2013	no statistically significant difference in p53
El-Domyati et al., 2015	TGF-β increased 10.1 ± 1.4% versus 15.9 ± 2.3% at the end of treatment (p=0.012)
	newly synthesized collagen increased 11.3 ± 3.1 versus 16.1 ± 3.8 (p<0.05)
Gungor et al., 2014	the number of elastin fibers was slightly increased in the post-treatment samples
Lapii et al., 2017a	increase of the epithelial proliferative activity 19.05 ± 2.86% versus 31.79 ± 2.25% (p<0.05)
Lapii et al., 2017b	the thickness of epithelium increased 114.19 ± 17.31µm versus 187.83 ± 15.35 (p≤0.05)
	volume density of capillaries increased 1.8 ± 0.2% versus 2.9 ± 0.3 (p≤0.01)
	number of capillary profiles in test area 8.5 ± 0.63 versus 12.10 ± 1.07 (p≤0.05)
Gaspar et al., 2017	an increase in the number of blasts and the fibrillar components (no quantification)

D/ Other outcomes - main results – all human studies

Authors	Results
Orringer et al., 2011	IL-1β elevated16-fold versus baseline (p<0.05)
	IL-8 elevated 200-fold versus baseline (p<0.05)
	cJun elevated 13.5-fold versus baseline (p<0.05)
	JunB elevated 52-fold versus baseline (p<0.05)
	MMP-1 elevated 230-fold versus baseline (p<0.05)
	MMP-3 elevated 140-fold versus baseline (p<0.05)
	MMP-9 elevated 10.4-fold versus baseline (p<0.05)
	procollagen I mRNA elevated 2.8-fold versus baseline (p<0.05)
	procollagen III mRNA elevated 3.8-fold versus baseline (p<0.05)
	procollagen I protein elevated 5.5-fold versus baseline (p<0.05)
Kunzi-Rapp et al. 2006	temperature ex-vivo lasered 70°C versus control 20°C
	in-vivo lasered 32°C versus control 29°C
Lukac et al., 2010	skin surface temperatures difference dependably on setting up to 60°C
Fistonic et al., 2016	surface temperature (up to 50µm) of the introitus mucosa 40-70°C dependably on mode and handpiece
Gaspar et al., 2017	maturation value baseline 20.8 ± 5.4 versus lasered 52.2 ± 8.5 (p<0.001)
	pH value 5.0 ± 0.4 versus lasered 4.4 ± 0.6 (p<0.001)

Table 3: Setting of lasers used in the reviewed studies. Listed companies are Coherent (Santa Clara, CA), Fotona (Ljubljana, Slovenia), Candela Corp (Wayland, MA), Wave-Light Laser Technologie AG (Erlangen, Germany)

Er:YAG Laser settings

Authors	Laser	Company	Mode	Fluence (J/cm2)	Pulses per packet	Pulse duration (ms)	Passes	Spot size (mm)	Repetition rate (Hz)
Tissue culture model									
Kao et al., 2003	UltraFine	Coherent	sub-ablative	0.8	5 or 10	-	single	4	5
Animal (rat)									
Majaron et al., 2000	UltraFine	Coherent	sub-ablative	1.4+/-0.2	1-10	-	single	4	10,33
Majaron et al., 2001	Fidelis	Fotona	-	1.3-5.2	10	0,15 or 0,55	single	3	20
Human									
Orringer et al., 2011	SmoothPeel	Candela Corp	microablative	0.75	-	-	single	5	-
Drnovsek-Olup et al., 2004	Fidelis M320A	Fotona	smooth	0.75 -2.00	6	250 (6x0,55)	single	5	20
Kunzi-Rapp et al., 2006	SupErb XL or BURANE XL	Wave-Light Laser Technologie AG	thermal (sub-ablative)	2.1 and 3.1	9–11	200–270	1,3,5	5	3
El-Domyati, et al., 2012	SkinPlus	Fotona	sub-ablative	2 – 3	-	200–250	-	-	-
El-Domyati et al., 2013	SkinPlus	Fotona	thermal (sub-ablative)	2 – 3	-	200–250	-	-	-
El-Domyati et al., 2015	SkinPlus	Fotona	thermal	2–3	-	200–250	-	-	-
Fistonic, et al., 2016	XS Dynamis, IncontiLase)	Fotona	smooth	3,6,10	6	250 (6x0,30)	several	7	1.6
Lukac, et al., 2010	XS Dynamis	Fotona	smooth	2	-	250	single	2-10	-
Lapii et al., 2017a	IncontiLase	Fotona	smooth	-	-	-	-	-	-
Lapii et al., 2017b	IncontiLase	Fotona	smooth	-	-	-	-	-	-
Gaspar et al.,2017	XS Dynamis, RenovaLase	Fotona	smooth	-	-	-	-	-	-
Gungor et al., 2014	-	-	smooth	3.2	-	250	single	7	-

Table 4: Readouts used within reviewed studies irrespective of skin and vagina.

Methodology	Specific method/stain	Target	Study
Histology	H&E	Tissue structure, thermal injury	(Majaron *et al.*, 2000; Majaron *et al.*, 2001; Kao *et al.*, 2003; Drnovšek-Olup *et al.*2004; Kunzi-Rapp *et al.*, 2006; El-Domyati *et al.*, 2012; Gungor *et al.*, 2014; Gaspar *et al.*, 2017; Lapii *et al.*, 2017a; Lapii, *et al.*2017b)
	Alcian blue, Masson's trichrome	Collagen	(Kunzi-Rapp *et al.*, 2006; Gungor *et al.*, 2014)
	Van Gieson, Orcein	Elastin	(El-Domyati *et al.*, 2012; Gungor *et al.*, 2014; Lapii *et al.*, 2017a; Lapii *et al.*, 2017b)
	PAS	Glycogen	(Lapii, *et al.* 2017b)
	Picosirius red	Collagen (newly synthetized)	(El-Domyati *et al.*, 2012, 2015)
Immuno-	Keratin 16	Epidermal injury	(Orringer *et al.*, 2011)
	Ki67	Cell proliferation	(Drnovšek-Olup *et al.*, 2004; Orringer *et al.*, 2011; Lapii *et al.*, 2017a)
Histochemistry	p53	Proliferation versus DNA repair	(El-Domyati *et al.*, 2013)
	Laminin γ, collagen IV, collagen VII	Basal membrane	(Drnovšek-Olup *et al.*, 2004; Orringer *et al.*, 2011; El-Domyati *et al.*, 2012)
	Collagen I, III, elastin, tropoelastin	Extracellular matrix	(El-Domyati *et al.*, 2012)
	CD68	Monocyte/ macrophage	(Drnovšek-Olup *et al.*, 2004)
	α-SMA, procolagen I	Myofibroblasts	(Drnovšek-Olup *et al.*, 2004; Kunzi-Rapp *et al.*, 2006)
	TGF-β	Cytokine	(El-Domyati *et al.*, 2015)
	IL-1β, IL-8	Cytokine	(Orringer *et al.*, 2011)
	AP-1 (cJun, JunB)	Transcription factor	(Orringer *et al.*, 2011)
RNA (qPCR)	Procollagen I,III	Collagen precursors and metalloproteinases	(Orringer *et al.*, 2011)
	MMP 1,3,9		
Other outcomes	Temperature measurement		(Kunzi-Rapp *et al.*, 2006; Lukac *et al.*, 2010; Fistonic *et al.*, 2012)
	Optical coherence tomography		(Kunzi-Rapp *et al.*, 2006)
	Maturation index		(Gaspar *et al.*, 2017)
	pH		(Gaspar *et al.*, 2017)

SUPPLEMETARY MATERIAL:

Appendix 1: Search strategy:

Pubmed 7.1.2018

"Mucous Membrane"[Mesh] OR mucous membrane*[tiab] OR mucosa[tiab] OR mucosal tissue*[tiab] OR "lamina propria"[tiab] OR "muscularis mucosae"[tiab] OR "laryngeal epithelium"[tiab] OR "nasal epithelium"[tiab] OR "Palate"[Mesh] OR palate*[tiab] OR uvula[tiab] OR "Periodontium"[Mesh] OR periodontium*[tiab] OR gingiva[tiab] OR "Vagina"[Mesh] OR vagina*[tiab] OR "Vulva"[Mesh] OR "Vulva"[tiab] OR clitoris[tiab] OR "Urethra"[Mesh] OR urethra*[tiab] OR Urinary Sphincter*[tiab] OR Urethral Sphincter*[tiab] OR Vesical Sphincter*[tiab] OR "Urinary Bladder"[Mesh] OR Bladder*[tiab] OR "Anal Canal"[Mesh] OR anal[tiab] OR anus[tiab] OR "Esophagus"[Mesh] OR Esophagus[tiab] OR oesophagus[tiab] OR "Skin"[Mesh] OR skin[tiab] OR dermis[tiab] OR epidermis[tiab] OR "Collagen"[Mesh] OR collagen*[tiab] OR procollagen[tiab] OR tropocollagen[tiab] OR "Elastin"[Mesh] OR elastin[tiab] OR tropoelastin[tiab] OR "Extracellular Matrix Proteins"[Mesh] OR "Extracellular Matrix Proteins"[tiab]

AND

"Lasers, Solid-State"[Mesh] OR Solid-State Laser*[tiab] OR Er-YAG[tiab] OR "Yttrium Aluminum Garnet"[tiab] OR Erbium-YAG[tiab] OR YAG[tiab]

Embase 7.1.2018

'mucosa'/exp OR 'mucosa*':ti,ab OR 'mucosae':ti,ab OR 'mucous':ti,ab OR 'mucus':ti,ab OR 'conjunctiva':ti,ab 'endometrium':ti,ab OR 'lamina propria':ti,ab OR 'palate'/exp OR 'palate*':ti,ab OR 'uvula*':ti,ab OR 'palatum':ti,ab OR 'periodontium'/exp OR 'periodontium':ti,ab OR 'gingiva':ti,ab OR 'vagina'/exp OR 'vagina*':ti,ab OR 'hymen':ti,ab OR 'vulva*':ti,ab OR 'urethra'/exp OR 'urethra*':ti,ab OR 'bladder sphincter':ti,ab OR 'lower urinary tract':ti,ab OR 'bladder'/exp OR 'bladder':ti,ab OR 'vesica urinaria':ti,ab OR 'anal canal'/exp OR 'anal':ti,ab OR 'analis canalis':ti,ab OR 'anus':ti,ab OR 'esophagus'/exp OR 'esophagus':ti,ab OR 'gastroesophageal junction':ti,ab OR 'skin'/exp OR 'skin':ti,ab OR 'cutis':ti,ab OR 'derma':ti,ab OR 'dander':ti,ab OR 'dermis':ti,ab OR 'epidermis':ti,ab OR 'scalp':ti,ab OR 'wrinkle*':ti,ab OR 'collagen'/exp OR 'collagen*':ti,ab OR 'procollagen':ti,ab OR 'tropocollagen':ti,ab OR 'elastin'/exp 'elastin':ti,ab OR 'tropoelastin':ti,ab OR 'extracellular matrix'/exp OR 'extracellular matrix':ti,ab OR 'basement membrane':ti,ab OR 'reticular lamina':ti,ab

'solid state laser'/exp OR 'solid state laser*':ti,ab OR 'Er-YAG':ti,ab OR 'Yttrium Aluminum Garnet':ti,ab OR 'erbium-YAG laser':ti,ab OR 'YAG':ti,ab

Cochrane 7.1.2018

mucous membrane OR mucosa OR mucosal tissue OR lamina propria OR muscularis mucosae OR laryngeal epithelium OR nasal epithelium OR palate OR uvula OR periodontium OR gingiva OR vagina OR Vulva OR clitoris OR urethra OR Urinary Sphincter OR Urethral Sphincter OR Vesical Sphincter OR Bladder OR anal OR anus OR Esophagus OR skin OR dermis OR epidermis OR collagen OR procollagen OR tropocollagen OR elastin OR tropoelastin OR Extracellular Matrix Proteins

Solid-State Laser OR Er-YAG OR Yttrium Aluminum Garnet OR Erbium-YAG OR YAG

Web of Science 7.1.2018
mucous membrane* OR mucosa OR mucosal tissue* OR lamina propria OR muscularis mucosae OR laryngeal epithelium OR nasal epithelium OR palate* OR uvula OR periodontium* OR gingiva OR vagina* OR Vulva OR clitoris OR urethra* OR Urinary Sphincter* OR Urethral Sphincter* OR Vesical Sphincter* OR Bladder* OR anal OR anus OR Esophagus OR skin OR dermis OR epidermis OR collagen* OR procollagen OR tropocollagen OR elastin OR tropoelastin OR Extracellular Matrix Proteins

Solid-State Laser* OR Er-YAG OR Yttrium Aluminum Garnet OR Erbium-YAG OR YAG

Chapter 5

LASER therapy for urinary incontinence and pelvic organ prolapse: a systematic review

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ABSTRACT:

Background: LASER therapy is now being proposed for the treatment of pelvic organ prolapse (POP) and urinary incontinence (UI).

Objectives: To systematically review the available literature on LASER therapy for POP and UI.

Search Strategy: Pubmed, Web Of Science and Embase were searched for relevant articles, using a three concept (POP, UI, LASER therapy) search engine composed as (concept 1 OR concept 2) AND concept 3.

Selection Criteria: Only full text clinical studies in English.

Data Collection and Analysis: Data on patient characteristics, LASER setting, treatment outcome and adverse events were independently collected by two researchers. Due to the lack of methodological uniformity meta-analysis was not possible and results are presented narratively.

Main Results: Thirty one studies recruiting 1530 adult women met the inclusion criteria. All studies showed significant improvement either on UI, POP or both, however the heterogeneity of LASER settings, application and outcome measures was huge. Only one study was a randomized controlled trial, two studies were controlled cohort studies. All three were on UI and used standardized validated tools. The risk of bias in the RCT was low on all seven domains; the controlled studies had a serious risk of bias. No major adverse events were reported, mild pain and burning sensation were the most common described adverse events.

Conclusions: All studies on vaginal and/or urethral LASER application for POP and UI report improvement, but the quality of studies needs to be improved.

Funding: none

Keywords: LASER, pelvic organ prolapse, urinary incontinence

'Tweetable abstract': There is weak evidence that LASER therapy is effective for urinary incontinence and pelvic organ prolapse #LASER#UI#POP

INTRODUCTION:

Around 30% of adult women have urinary incontinence (UI)[10] and 5-10% have pelvic organ prolapse (POP)[4, 5]; both negatively impacting quality of life[205, 206]. Treatment can be either conservative or surgical, each with its specific advantages and disadvantages. Conservative treatment typically includes life style interventions, physiotherapy and/or the use of pessaries[207]. Pelvic Floor Muscle Therapy (PFMT) and behavioral training are often combined and are safe and cheap options with a low risk for adverse events (AE). Potential harm of PFMT is caused by incorrect training, which may lead to worsening of symptoms or the occurrence of pain[14]. PFMT may be cost-effective but further research is needed as well as on the long-term effectiveness[208]. Pessaries often relieve prolapse symptoms as good as PMFT and are cost effective[209]. They may however cause symptoms like bleeding, extrusion, severe vaginal discharge, pain and constipation, in up to 60% of long term users. This may eventually lead to their discontinuation[15, 16]. Several drugs may be used for UI; depending on the type, this may include anticholinergics or a beta-3 agonist (for urge UI) or serotonin reuptake inhibitors (for stress UI). All may cause significant AE, which may, again, lead to their discontinuation[17].

Surgery is an alternative for selected women with UI and POP. In turn, surgery may have complications and/or AE, some of them difficult to treat. When prosthetic materials are used, there may in addition be graft related complications (GRC)[22]. Though the use of prosthetic materials is now restricted (in some countries suspended), the occurrence of GRC has caused concern among perspective patients[22, 23, 210]. Therefore women may consider alternative options.

Adopted from dermatology, two LASER types - erbium-doped yttrium aluminium garnet (ER: YAG) and carbon dioxide (CO_2) - were introduced as a novel, non-invasive treatment in gynecology, first for genitourinary syndrome of menopause (GSM) and later for POP and UI. Manufacturers are claiming that vaginal and/or intra-urethral LASER applications are an alternative to surgery and/or pharmacotherapy, offering similar or better effects with minimal AE[42, 43]. The use of LASER is spreading quickly in hospitals and private practices. Prices for a single application may be as high as 1000€[44]. At this moment, that seems inversely related to the level of evidence on the safety and efficacy of their use for those given indications[211]. Herein, we aimed to systematically review the literature on the efficacy and safety of Er:YAG and CO_2 LASER therapy for POP and UI.

MATERIAL AND METHODS

Reporting

The reporting of this systematic review is done in line along the PRISMA-guidelines (Preferred Reporting Items for Systematic reviews and Meta-analyses)[212].

Protocol and registration

The following research question was addressed: 'What is currently known on LASER therapy in women with urinary incontinence and/or pelvic organ prolapse'. This systematic review and its protocol were registered and published at the National Institute for Health Research PROSPERO, International Prospective Register of Systematic Reviews https://www.crd.york.ac.uk/PROSPERO, registration number: CRD42019132229 on 16/07/2019.

Eligibility criteria

Only original studies in English were included on subjects who were women diagnosed with urinary incontinence and/or pelvic organ prolapse. Animal, cadaveric and ex vivo studies were excluded as well as women with other diagnoses than POP and/or UI (i.e. cervical dysplasia, anal fistula, kidney stones…) and studies in men.

Furthermore, only studies with LASER therapy as the intervention type were included, hence excluding radiofrequency and other energy-based modalities. Primary outcome measures had to be clinical.

Information sources

Relevant literature was extracted from three electronic databases: Pubmed, Web Of Science and Embase. The search strategy was primarily designed for the database Pubmed, consisting of relevant MeSH-terms (Medical Subject Headings terms) and synonyms with Title/Abstract filter. Afterwards, Emtree terms, analogous to MeSH-terms, were selected in Embase. Applicable new terms and synonyms were added as synonyms with Title/Abstract filter to the search strategy for Pubmed as well. The final search was converted to a search strategy compatible with Web Of Science. The search was performed on the 14th of April 2019 using a three concept (POP, UI, LASER therapy) search engine composed as (concept 1 OR concept 2) AND concept 3. Details of the search strategy can be found in Appendix S1.

Study selection

All search results were screened in two phases. First, two reviewers (KM, LVD) independently screened all articles on title and abstract in Endnote (Endnote X8, Clarivate Analytics, Philadelphia, US). In case of lack of consensus, the full text article was included in the second phase. Again, both reviewers checked whether the selected full text papers met the inclusion criteria. In the absence of consensus, a third opinion (JDP) was asked.

Data collection process

From the included trials, data were gathered in three data extraction tables. For the main table, data were extracted on characteristics of trial participants, including diagnosis, and setting. A second table contained outcome measures and results. The third table was on information on LASER type, LASER settings and treatment protocol. Each researcher (KM, LVD) performed data extraction on half of the included articles and checked the data extracted by the other researcher on the other half of articles. Therefore, both researchers eventually went through the full paper.

Risk of bias

The Cochrane Collaboration's tool and The Risk Of Bias In Non-randomized Studies – of Interventions (ROBINS-I) assessment tool (version for cohort-type studies) were used to assess risk of bias and study quality.

Data synthesis and Statistics

The lack of methodological uniformity in the included articles does not allow performing a meta-analysis. For this reason, results are presented as a narrative review with clinical outcomes as primary outcome measure and adverse events as secondary outcome measure. Core outcome sets for female pelvic floor disfunction including urinary incontinence and pelvic organ prolapse are currently developed by Doumouchtsis and his team[213]. Awaiting these publications, all clinical outcomes used in the studies were included

Funding: The study received no funding.

Patient and public involvement: Any patients or public were involved in this research work.

RESULTS

Study selection

A total of 31 studies were identified (Fig. 1). Our search provided 2279 citations. After adjusting for duplicates, 1507 articles were screened on title and abstract, allowing us to exclude 1452 studies for the reasons displayed in Figure 1. We examined 55 full text papers, of which eventually 24 studies did not meet the criteria for population or outcome or design. Checking the references of relevant papers or searching for studies that cited the papers selected, did not identify additional material.

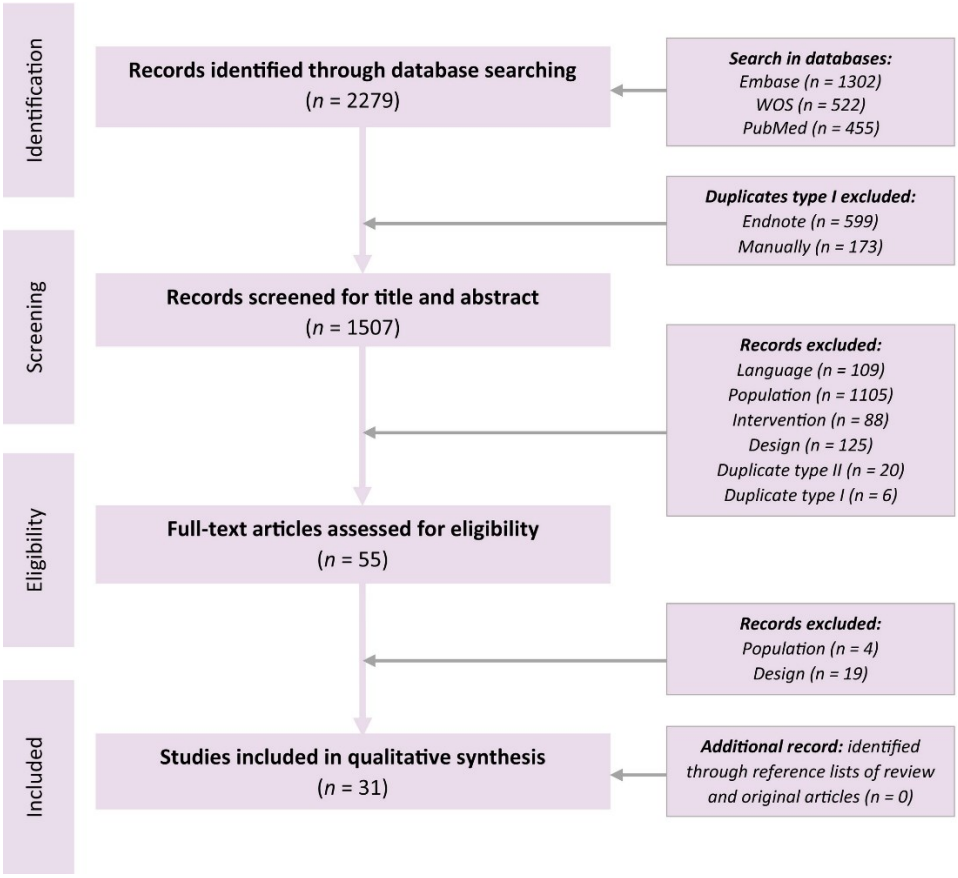

Figure 1: PRISMA flow diagram

Study characteristics:

Methods

Thirty were cohort studies and one was a randomized controlled trial[214]. All studies were written in English and published between 2015 and 2019. Most were unicentric and originating from Italy (n = 5), Taiwan (n = 4), Slovenia (n = 3), Croatia (n = 3), Greece (n = 2), Australia (n = 2), Argentina (n = 2), Germany (n = 2), Japan (n = 2), Saudi Arabia (n =1), Iraq (n = 1), Colombia (n = 1), Iran (n = 1), Chile (n = 1), USA (n = 1).

Participants

The studies involved 1530 adult female participants, on average 55 participants per study (min. 16; max. 175). Primary diagnosis at the point of inclusion was SUI (n = 15 studies), Overactive bladder (OAB) (n = 2), POP (n = 2), and 12 studies involved women with GSM, in whom the clinical effect on POP (n = 2) and UI (n = 11) was a secondary outcome. The main inclusion criteria and patient characteristics are listed in Table S2, exclusion criteria in Table S1.

Intervention

The LASER types used were either ER: YAG (n = 21) or CO_2 LASER (n = 9), or both, yet the outcomes are reported separately[215]. The LASERs were used mainly vaginally (29 studies) and on the vulva (n = 21) and/or urethra (n = 2)[216, 217]. Settings of the LASERs varied, however some authors did not mention the LASER settings[218-225]. Table S3 and S4 displays the LASER setting details. In the only RCT[214] the sham group was treated with the same procedure but with zero intensity settings, without receiving therapeutic irradiation. There were two controlled cohort studies[222, 223], with either surgery (midurethral sling[222]) or pharmacotherapy (anticholinergics and β-agonist) as a comparator[223].

Outcomes

Primary outcome

Primary outcome measure for SUI studies was in most cases the ICIQ-UI SF questionnaire[226] and the 1-h pad test. For women with POP, the primary outcome was the POPDI6[227] questionnaire and the Baden-Walker scale[228]. For women with OAB, the OABSS questionnaire was used[229]. Other questionnaires and methods to evaluate outcomes are listed in Table S5.

Secondary and additional outcomes

Thirteen studies described the effect of LASER on sexual functioning with either questionnaires (FSFI (n=6), PISQ12 (n = 3), both questionnaires (n= 1)) or self-reporting sexual gratification (n=2) as outcome tool[230] (Table S5). Side effects of LASER treatment were reported in 17 studies and usually judged categorized as mild to moderate by authors (Table 1).

Table 1. Adverse events as reported in studies.
Legend: AE, adverse events; NR, not reported.

	Complications	Number of studies	Number of participants	Number of AE (%)	Reference
During application	Pain/ discomfort	7	409	NR	*Alfarra et al. (2018), Fistonić et al. (2016), Lin YH et al. (2017), Ogrinc et. al. (2015), Ogrinc et. al. (2017), Pardo et al. (2016), Samuels et al. (2019)*
	Burning sensation	4	120	NR	*Alfarra et al. (2018), Gambacciani et al. (2015; VEL), Lin YH et al. (2017), Lin KL et al (2019)*
	Warmth sensation	3	161	NR	*Blaganje et al. (2018), Fistonić et al. (2015), Fistonić et al. (2016)*
	Irritation of introitus	2	157	NR	*Lin HY et al. (2018), Fistonić et al. (2015), Pitsouni et al. (2016)*
Immediate Rx responses	Vulvar edema	3	144	NR	*Samuels et al. (2019), Fistonić et al. (2015), Fistonić et al. (2016)*
	Erythema	1	40	NR	*Samuels et al. (2019)*
	Bleeding	2	69	6 (8.7%)	*Gaspar et al. (2018), Samuels et al. (2019)*
	Dysuria	2	51	6 (11.8%)	*Gaspar et al. (2017), Gaspar et al. (2018)*
	Tissue retraction	1	40	1 (2.5%)	*Samuels et al. (2019)*
Next few days after Rx	UTI	4	260	7 (2.7%)	*Behnia-Willison et al. (2017), Behnia-Willison et al. (2019), Gaspar et al. (2018), Mothes et al. (2018b)*
	Dysuria	1	40	2 (5.0%)	*Samuels et al. (2019)*
	De novo urgency	3	305	10 (3.3%)	*Ogrinc et. al. (2015), Blaganje et al. (2018), Fistonić et al. (2015)*
	Recurrence of genital herpes	2	160	2 (1.2%)	*Behnia-Willison et al. (2017), Behnia-Willison et al. (2019)*
	Vaginal spotting/bleeding	5	259 41	6 (2.3%) NR	*Mothes et al. (2018b), Samuels et al. (2019), Behnia-Willison et al. (2017), Lin YH et al. (2017)* *Lin KL et al (2019)*
	Change in vaginal discharge	6	246 105	57 (23%) NR	*Behnia-Willison et al. (2017), Behnia-Willison et al. (2019), Blaganje et al. (2018), Lin YH et al. (2017)* *Fistonić et al. (2015), Fistonić et al. (2016)*
	Itching	2	70	9 (12.9%)	*Lin YH et al. (2017), Samuels et al. (2019)*
	Increased vaginal dryness	1	57	1 (1.7%)	*Blaganje et al. (2018)*
	Lower pelvic pain	2	124	4 (3.2%)	*Behnia-Willison et al. (2017), Gaspar et al. (2017)*
	Vulva discoloration	1	30	5 (16.7%)	*Lin YH et al. (2017)*

Results of individual studies

Results of and complications reported in individual studies are displayed in Table S5 and divided according to the initial diagnosis on entry into the study, being SUI, OAB, POP and GSM.

Risk of bias

According to the ROBINS-1 tool, all studies are at least at moderate risk of bias (n = 6 moderate, n = 17 serious, n = 5 critical and n = 2 unclear risk of bias). Bias assessment of the RCT with The Cochrane Collaboration's tool revealed low risk on all seven domains (Appendix S2). Six studies did not have a statement on conflict of interest/funding/ declared no funding neither a conflict of interest.

Nineteen declared they received no funding neither they did have a conflict of interest[217, 224, 231-236]. Eight declared financial support from the LASER company (LASER company provided the equipment, funded the study or authors of the study were employee of the company in the same time). Four articles did not mention whether they received funding neither whether there was a conflict of interest even though it was required by the journal.

Syntheses of results

Urinary incontinence

All studies reported significant improvement both for objective and subjective outcome measures.

Stress urinary incontinence is the best studied UI type in the articles included in this review, with the ICIQ-UI SF and the 1-h pad test respectively as the most frequently used outcome measure. In the single randomized sham-controlled study, the reduction on ICIQ-UI SF score was significantly higher ($p<0.001$) in the LASER than in to the sham group[214]. In the study that compared LASER therapy with tension free vaginal tape (TVT) or transobturator tape (TOT), similar improvements were found at one year follow-up based on the ICIQ-UI SF, OABSS as well as on the 1-h pad test ($p<0.001$)[222]. A one year follow-up study of Ogrinc et al. revealed a significant decrease in all 175 women on ISI (incontinence symptoms scores; $p<0.001$)[237], whereof 108 having no more UI. Also ICIQ-UI SF and APFQ scores remained significant lower at one year follow-up ($p=0.001$)[225], ($p<0.01$)[238]. Six other studies with SUI as primary diagnosis reported curing rates ranging from 38.1% – 75.5% at six months follow-up[216, 224, 239, 240] and age, baseline ICIQ-UI SF values and BMI as significant predictors of Rx efficacy[219, 241]. No difference in both LASER types were observed[215].

There were seven studies that had improvement in GSM as a primary outcome, yet also included women with SUI[217, 220, 232, 236, 242-244]. These studies also reported a significant reduction in the ICIQ-UI SF scores that was sustained at one year follow-up ($p<0.01$)[217, 242, 243]. On the longer term, that effect was no longer present at two year follow-up [244], yet González Isaza reported a positive effect up-to three years after treatment ($p<0.001$)[220].

There were three other GSM studies where women with unspecified incontinence were included showing significant improvement on the ICIQ-UI SF scores at four weeks follow-up ($p<0.001$)[234], at twelve months follow-up ($p<0.001$)[245] as well as a satisfaction rate of 75%[235].

Three studies addressed urge incontinence primarily[223, 246, 247], yet in one in combination with GSM[247]. A significant improvement in the ICIQ-OAB score was measured after the first treatment ($p<0.05$)[246] and lasted up-to 30 days post treatment ($p<0.0001$)[247]. There was no longer follow-up available. Okui et al. (2019) compared LASER with anticholinergics and ß3-adrenoceptor antagonist and treatment effects were comparable at one year follow-up, when measured by the OABSS questionnaire ($p<0.001$)[223].

Pelvic organ prolapse

There was one study primarily targeting women with POP. Ogrinc did a cohort study in women with cystocele[221] and reported an anatomical improvement to grade 0 or 1 in 85% and in the remaining 15% to grade II after two to five LASER therapy sessions. Lower cystocele grades were associated with a higher success rate. Only in 5% of women there was no improvement[221]. We could not identify a formal subjective improvement assessment.

Alkhafajy used three LASER applications in 90 women with "vaginal loosening", later on referred to as "vaginal wall prolapse" – yet no formal definition is referred to. Three weeks after the last application, women reported significant improvement in vaginal tightening sensation using a subjective three grade scale ($p<0.05$), as well as sexual function ($p<0.05$)[218].

Several studies on women with SUI- included women with POP grade II, yet only one cohort study reported subjective improvement in POPDI-6 scores six months after three applications ($p<0.001$)[240].

Complications

Complications found in the reviewed articles were mild and transient, grade I-II according to Dindo classification[248], with no major adverse events reported (n=1530 patients; 4351-5072 applications). At the time of LASER application, a mild pain (VAS ranging from 0 – 5[221, 225, 231, 234, 237, 249]), burning[225, 235, 240, 243, 249] and warmth sensation[214, 219] were commonly reported. For none of these outcomes, specific number of patients were given. After LASER application, the most frequent adverse event was increased vaginal discharge, lasting up to three weeks and requiring treatment in some (n=6) cases[214, 219, 225, 238, 250]. Only seven patients (0.47%) reported urinary tract infection (UTI) after treatment[217, 225, 231, 232, 238, 250], of which three were considered as postcoital UTI[250](Table 1).

Sexual function

Sexual function was a secondary outcome in 13 out of 31 included studies. In seven studies the primary diagnosis was SUI. In three a significant improvement were reported in FSFI-scores (Blaganje, 2018, p=0.025; Alfarra, 2018, p-value is not given)[214, 249], in PISQ-12 scores ($p<0.01$)[214] or in self-reported "sexual gratification"[239]. Four studies, however, did not report a significant improvement in FSFI[215, 224] or PISQ-12 score[219, 225]. Mohajeri et al offered women with OAB LASER, and also reported that their sexual function was not improved (PISQ-12)[246]. Alkhafajy et al. (2018) observed improved sexual function using a subjective three grade scale in women with vaginal relaxation ($p=0.004$)[218]. In one study in women with GSM there was a significant improvement in FSFI-scores in all participants ($p<0.001$)[245] and in another in 35 out of 40 participants ($p<0.001$)[235]. In two other studies in women with GSM, there was a significant decline of dyspareunia (VAS score) ($p<0.05$)[234, 236].

DISCUSSION:

Main Findings

This review covers data on 1530 patients, from 15 different countries, who underwent together an estimated 4351 (minimum) to 5072 (maximum) LASER sessions. One or two LASER devices (Er:YAG/CO2) using vulvar, urethral and/or vaginal probes were used either for UI and/or POP. We identified one randomized double blinded sham-controlled study in women with SUI. Blaganje et al. (2018) showed a significant reduction in the ICIQ-UI SF after Er:YAG LASER therapy versus sham manipulated (-3.86 points; CI -5.06 to-2.66; $p <0.001$). However, only 21% were subjectively dry (ICIQ-SF = 0) versus 4% of sham operated patients ($p = 0.006$; Risk Ratio (RR) = 6.00, 95%CI: 1.41–25.59). The overall low subjective cure rate may be because only one LASER session was used, and better results could have been observed if additional sessions were given. Of relevance, this study had a placebo group, though the effect was limited (4%). Weaknesses were the short follow-up period (three months) and the single LASER session, as well as the fact that objective measures were not used[214].

Two additional controlled cohort studies were identified. Firstly, Okui et al (2018) compared three Er:YAG LASER sessions to one of the two sling methods, e.g. either TVT or TOT in women with SUI. Based on ICIQ-UI SF scores and pad testing, LASER therapy had a comparable effect ($p<0.001$) to surgery at 12 months follow-up. The percentage of "dry patients" (urine loss=0 g) in the TVT, TOT, and LASER groups was 69, 68, and 50%, respectively (not significant)[222]. In a later study, Okui et al. (2019) compared three Er:YAG LASER applications to two standard drugs (4 mg fesoterodine, 25 mg mirabegron) in women with OAB (excluding neurogenic OAB). Based on the OABSS score, LASER had a comparable outcome to both medically treated groups ($p < 0.001$)[223] at 12 months. In other words, in both controlled studies LASER had an effect that was comparable to the standard or care, and without adverse events. However, both studies have a serious risk of bias, caused by neither proper classification of intervention (LASER settings are missing) nor proper measurements of outcome (missing blinding)[222, 223].

The remaining 28 articles (90%) were cohort studies, all reporting improvement. These studies have however several methodological shortcomings. In 75% (21/28) of studies, no power analysis was made. In 71% (20/28) the primary outcome was not clearly defined, and in 14% (4/28) no standardized method endorsed was used. Details on LASER settings were not available in 21% (6/28), and number of LASER sessions ranged widely (one to five). Outcomes were assessed at a variable number of timepoints (range: 1-7) and follow-up ranged between three weeks and 36 months. In 72% of studies, there was a serious or critical risk of bias in 72% (20/28), and in two (7%), bias assessment was not possible. In two studies (Gaspar et al. 2017 and Pardo et al. 2016) estrogen creams were used prior the LASER application in some patients (women with atrophy[239] or "when needed"[216]) without referring to the number of patients or differences in outcomes.

Many studies mention minimal side effects (burning, warmth, irritation or pain) during application, but usually without numeric details. In terms of major adverse events, like death, organ failure or surgical intervention (Dindo classification III-V)[248] none during or after were reported (>4351 applications). Conversely, mild post-application adverse events (Dindo classification I-II)[248], of which the most frequent were change in vaginal discharge, urgency, transient edema, itchiness, urinary tract infection, vaginal bleeding, and others (Table 1) were reported. When taking account only properly addressed AE in post treatment period, AE were occurring in 6.7% of patients (116/1711).

Strengths and Limitations

This review has several strengths and weaknesses. With a comprehensive search strategy, using three main repositories, we made sure no article on our topic was neglected. We have attempted to systematically and clearly display all analyzed outcomes, however this was not easy for two different diagnoses. Another potential limitation is that only articles published in English were included.

Interpretation

The lack of methodological uniformity did preclude performing a meta-analysis, so our results are presented as a narrative synthesis. Our review identified the up till now, first and only RCT in women with SUI. Together with one controlled cohort study these studies demonstrated benefit of LASER therapy for SUI. In one other controlled cohort study the same was shown for OAB. There are much less studies in women with POP, and they are not of good quality or outcomes cannot be well compared. This review covers >4351 LASER applications and suggests that LASER application in the short term may cause local side effects, however mild and transient in nature. Current studies do not allow evaluating long term complications, such as stenosis, fibrosis etc. While these may potentially occur and are not yet identified because of the recent introduction of this technology.

CONCLUSSION:

LASER therapy seems to have a beneficial effect on prolapse and urinary incontinence, yet the level of evidence remains low. In terms of short term safety, no major problems seem to emerge, not during nor after the

application. There is a range of minor side effects, a problem that requires more research to exclude their persistence. Regarding long term safety, there is currently no information available. There is definitely room for proper randomized multicentric studies comparing LASER with standard treatments. Future studies should also address cost-effectiveness, in particular looking on a longer term perspective, as, for instance, surgical results may have more longevity than LASER, which may require re-application. It is impossible to say at this moment what LASER type should be used.

Disclosure of Interests

Nothing to declare.

Contribution to Authorship

JDP and LK conceived the study. LVD and KM performed search, independently screened and selected eligible studies, independently performed risk of bias assessment and completed data extraction, verifying each other work. JDP, LVD, KM, AP, IG, LK contributed to the interpretation of the data and the writing the article. All authors reviewed and approved final version and accept responsibility for the paper as published.

Details of ethics approval

Not applicable.

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SUPPLEMENT:

Table S1. Exclusion criteria.

Legend: 1-h pad test, one hour pad test; BMI, Body mass index; d, Day; DM, Diabetes mellitus; g, Gram; HRT, Hormone replacement therapy; ICS, International Continence Society; kg/cm^2, Kilogram-Meter Squared; LUTI, lower urinary tract infection; m, Month; ml, millilitre; MUI, Mixed urinary incontinence; OAB, Overactive bladder; PAP smear, Papanicolaou test; PAP test, Papanicolaou test; PFM, Pelvic floor muscles; POP, Pelvic Organ Prolapse; POP-Q, Pelvic Organ Prolapse Quantification; pt, Patient; Rx, Treatment; UI, Urinary incontinence; UTI, Urinary tract infection; UUI, Urge urinary incontinence; VD, Vaginal delivery; w, Week; y, Year.

First Author (date)	Exclusion criteria
Alfarra et al. (2019)	Pregnancy; neuromuscular diseases; POP grade III and IV.
Alkhafajy et al. (2018)	Pregnancy; pelvic or vaginal wall infection; local injury; undiagnosed vaginal bleeding; active menstruation.
Anthanasiou et al. (2018)	Use of any nonhormonal or hormonal therapy within 3 or 6 months before the initiation of Rx; presence of active genital lesions; POP-Q ≥ 2.
Behnia-Willison et al. (2017)	Unexplained bleeding; abnormal PAP smear; active genital infections or any kind of active cancer within the urogenital area; receiving concomitant treatment throughout the study period.
Behnia-Willison et al. (2019)	POP-Q ≥ 2; acute or recurrent UTI; pregnancy; current malignancy; known cervical dysplasia; undiagnosed abnormal uterine bleeding.
Blaganje et al. (2018)	POP-Q ≥ 2; inability to perform correct PFM contraction; urgency or MUI; infection previous gynecologic surgery or irradiation.
Fistonić et al. (2015)	Severe POP and damage of the rectovaginal fascia; UUI; neurogenic bladder; severe neurological conditions associated with UI; insulin-dependent DM; actual UTI; hematuria; age ≤18 or >70y; pregnancy; <24 w after VD; BMI > 35 kg/m^2; intake of photosensitive drugs; injury or/and active infection in the treatment area; undiagnosed vaginal bleeding.
Fistonić et al. (2016)	Severe POP and damage of the rectovaginal fascia; UUI; neurogenic bladder; severe neurological conditions associated with UI; insulin-dependent DM; actual UTI; hematuria; age ≤18 or >70 y); pregnancy; <24 w after VD; BMI > 30 kg/m^2; intake of photosensitive drugs; injury or/and active infection in the treatment area; undiagnosed vaginal bleeding.
Fistonić et al. (2018)	Photosensitive disorder or use of photosensitizing drugs; severe cystocele, uterine prolapse, and damage of the recto-vaginal fascia (grade 2-3 POP-Q); UUI; severe neurological conditions associated with UI; neurogenic bladder; insulin-dependent DM; actual UTI; hematuria, age ≤18 or >70 y); pregnancy; <24 w after VD; BMI > 35 kg/m^2; injury or/and active infection in the treatment area; undiagnosed vaginal bleeding.
Gambacciani et al. (2015a	Vaginal lesions, scars, active or recent (30 d) infections of the genitouonary tract; abnormal uterine bleeding: use of vaginal preparations within the 30 d prior to the study: history of photosensitivity disorder or use of photosensitizing drugs; genital prolapse (POP-Q grade lll—IV); serious or chronic condition that could interfere with study compliance: treatment with hormones to relieve menopausal symptoms in the 12 m before Rx.
Gambacciani et al. (2015b)	Vaginal lesions, scars, active or recent (30 d) of the genitourinary tract infections; abnormal uterine bleeding; use of lubricants or any other local preparations, within the 30 d prior to the study; history of photosensitivity disorder or use of photosensitizing drugs; genital prolapse (POP-Q grade II – III); serious or chronic condition that could interfere with study compliance; treatment with hormones or other medicines to relieve menopausal symptoms in the 12 m before Rx.
Gambacciani et al. (2018)	Use of lubricants, local preparations, hormones or other medications to relieve menopausal symptoms in the previous 3 m prior to inclusion in the study; lesions, scars or infection, active or recent (30 d) of the genitourinary tract; abnormal uterine bleeding; history of photosensitivity disorder or use of photosensitizing drugs; genital prolapse (grade II–III classification POP-Q); and serious or chronic illness that could interfere with the study.

Gaspar et al. (2017)	Pt taking antihypertensive medication or alpha-blockers (e.g., prazosin, terazosin, etc.…) were excluded from the study, since these medications are known to produce relaxation of the rhabdosphincter. Pt treated with pelvic radiotherapy or pelvic surgery were also excluded from the study.
Gaspar et al. (2018)	POP-Q >I and BMI > 35 kg/m^2.
González et al. (2018)	POP-Q grade > I in the anterior compartment; pt who were not adequately classified because of previous surgery, recurrent LUTI or obesity (BMI >35 kg/m^2).
Lin YH et al. (2017)	Urinary tract infection; POP > grade II; pregnancy; hematuria; childbirth within one year; abnormal vaginal bleeding; damage of vaginal fascia; history of spinal cord injury; post radical hysterectomy; stroke; autoimmune disease; and 1-h pad test greater than 50 g.
Lin HY et al. (2018)	Pregnancy; poor controlled DM; gynecological cancer history; POP-Q grade > II; taking medications that caused photosensitivity; vaginal bleeding; or an infection in the area to be treated.
Lin KL et al. (2019)	Not mentioned.
Mohajeri et al. (2018)	Use of other OAB therapies (drug, pelvic floor muscle training, Botox injection) within the 3 months prior to the commencement of the study; POP grade III and IV; recent treatment with isotretinoin; presence of vaginal wound; an abnormal PAP smear; DM; BMI > 30 kg/m^2.
Mothes et al. (2018a)	Abnormal PAP test or gynecological examination finding; vaginal wounds or infections; UTI; pregnancy; menstrual period; intake of photosensitive drugs or topical estrogens and a previous history of malignant pelvic or systemic disease.
Mothes et al. (2018b)	Abnormal PAP test or gynecological examination finding; vaginal wound or infection; urinary tract infection; pregnancy; menstruation; current use of photosensitive drugs or topical estrogens; and previous history of malignant pelvic or systemic disease.
Ogrinc et. al. (2015)	Pregnancy; intake of photosensitive drugs; injuries or vaginal bleeding; infection in the treated area and an existence of pure UI.
Ogrinc et. al. (2017)	POP surgery; pregnancy; intake of photosensitive drugs; vaginal bleeding injuries or infection in the treated area.
Okui et al. (2018)	No objective evidence of cardiovascular disease, a history of surgery for UI or a history of treatment with drugs for UI; overactive bladder; neurogenic bladder dysfunction; estrogen therapy; cystocele; uterine prolapse; rectocele; UUI; neuropathy such as spinal stenosis. When posed greater hindrance to daily life than did SUI, drugs that were not permitted in the study were administered during the preoperative observation period, and the subject was excluded. Women who were pregnant, breastfeeding, or who wanted to become pregnant were excluded.
Okui et al. (2019)	Undergoing any other OAB treatment; a history of female hormone replacement therapy or botulinum toxin injection therapy; SUI; the presence/absence of brain, spinal, and peripheral nerve diseases was verified, and patients with neurogenic OAB were excluded; gynecological diseases in the adjacent organs (e.g., uterine cancer); POP grade > 0 and those with a history of surgery for the same were excluded.
Pagano et al. (2017)	Active genitourinary cancer; active genitourinary infections; UUI and MUI; POP > grade II and history of anamnestic allergic reactions to laser energy.
Pardo et al. (2016)	Exclusive UUI; severe prolapse; pregnancy; previous surgery due to treated condition; patients with severe neurological conditions; vaginal lesions; genitourinary tract infections; abnormal vaginal bleeding; history of photosensitivity disorder or use of photosensitizing drugs and hematuria.
Perino et al. (2016)	Clinically significant bladder outflow obstruction; significant post-void residual volume (>200 ml); associated SUI; diabetic neuropathy; use of concomitant UI medications; symptomatic UTI; active genital infections; previous pelvic radiation therapy; or previous or current malignant disease of the pelvic organs, POP grade >II (according to the Half Way System for the quantification of POP) and/or the use of HRT (systemic or local) up to 6 m before the study recruitment period; use of psychotropic drugs.

Pitsouni et al. (2016)	Use of any form of hormone therapy (systemic or local) within the previous 6 m; use of lubricants or vaginal moisturizers within the last month; active genital infections, POP-Q ≥II and any disease that would interfere with compliance to the protocol.
Samuels et al. (2018)	Previous vaginal reconstructive surgery or treatment for vaginal tightening within the past 12 m; previous laser or RF treatment within the prior 6 m; POP ≥II, according to ICS-POP-Q system; acute or recurrent UTI; active genital infections; undiagnosed vaginal bleeding; anticoagulation medications 1 w prior to and during the treatment course; currently using immunosuppressive medications or use of systemic corticosteroid therapy 6 m prior to and throughout the course of the study; suffering from hormonal imbalance or any serious disease or chronic condition that could interfere with study compliance excluded participation in the study.
Tien et al. (2016)	Women who had never been sexually active and those with undiagnosed abnormal vaginal bleeding were excluded.

Table S2. Characteristics of included studies.

Legend: Values are expressed in the age column as mean ± SD, median (minimum – maximum) or median (IQR) depending on normality of the data; Post Rx means after last therapy session. Abbreviations: 1-h pad test, one hour pad test; APFQ, Australian Pelvic Floor Questionnaire; CO_2, Carbon dioxide laser; d, Day; DM, Diabetes mellitus; Er: YAG, Erbium-doped yttrium aluminium garnet laser; FSFI, Female Sexual Function Index; gr, Grade; GSM, Genitourinary syndrome of menopause; ICIQ-FLUTS, Questionnaire for evaluating female lower urinary tract symptoms and impact on quality of life; ICIQ-UI SF, International Consultation on Incontinence Questionnaire - Urinary Incontinence short form; ICS, International Continence Society; IIQ-7, Short form of the Incontinence Impact Questionnaire; IQR, Interquartile range; ISI, International severity index; KHQ , Kings` health questionnaire; min, Minimum; max, Maximum; m, Month; MUI, Mixed urinary incontinence; NR, Not reported; NSR, 10-point numerical scale response; OAB, Overactive bladder; OABSS, Overactive Bladder Symptom Score; OAB-Q SF, Overactive Bladder Questionnaire - short form; PGI-I, Patients Global Impression of Improvement; pH, Power of hydrogen; PISQ-12, Short form of the Pelvic Organ Prolapse/Urinary Incontinence Sexual Questionnaire; POP, Pelvic Organ Prolapse; POPDI-6, Pelvic Organ Prolapse Distress Inventory; POP-Q, Pelvic Organ Prolapse Quantification; PPBC, Patient perception of Bladder condition; pt, Patient; RCT, Randomized controlled trail; Rx, Treatment; SUI, Stress urinary incontinence; TOT, Transobturator tape; TVT, Tension free vaginal tape; UDI-6, Urogenital Distress inventory; UI, Urinary incontinence; US, Ultrasound; USS, Urgency severity scale; UTI, Urinary tract infection; VAS, Visual analogue scale; VHI, Vaginal health index; VMV, Vaginal maturation value; w, Week; y, Year.

First author, Country (date)	Laser Type & brand	PATIENT CHARACTERISTICS Number	Control group	Age (years)	Menopausal	Diagnosis	Inclusion criteria UI	Inclusion criteria POP	OUTCOME MEASURES Primary (timepoints)	OUTCOME MEASURES Secondary (timepoints)
RCT										
Blaganje et al. Slovenia (2018)	ER: YAG XS Dynamics, Fotona, Ljubljana, Slovenia	57	57 (sham)	35.95 ± 6.36 41.84 ± 5.67	0%	SUI only	Urodynamics	Max POP-Q gr I	ICIQ-UI SF (Baseline, 3 m post Rx)	Perineometry, PISQ-12, FSFI (Baseline, 3 m post Rx)
Controlled cohort study										
Okui et al. Japan (2018)	ER: YAG Fotona Smooth™ XS, Fotona, Ljubljana, Slovenia	50	50 (TVT) 50 (TOT)	50.3 ± 13.2 48.7 ± 13.9 47.8 ± 13.9	NR	SUI and MUI	NR	NR	1-h pad test, ICIQ-UI SF (Baseline, 12 m post Rx)	OABSS (Baseline, 12 m post Rx)
Okui et al. Japan (2019)	ER: YAG Fotona Smooth™ XS, Fotona, Ljubljana, Slovenia	50	50 (fesoter-odin) 50 (mirabe-gron)	63.8 ± 2.56 63.9 ± 2.76 65.32 ± 2.28	NR	OAB only	Symptoms OAB	POP-Q gr > 0 excluded	OABSS (Baseline, 12 m after 1st Rx)	VHI (Baseline, 12 m after 1st Rx)
Cohort Study										

Alfarra et al. Saudi Arabia (2018)	ER: YAG Action II petite lady, Lutronic, Goyang, South Korea	30	No	25-55	NR	SUI and sexual dysfunction	Urodynamics	POP-Q gr I or II	Bladder diary, PFX2 (Baseline, 1, 2 m post Rx)	FSFI (Baseline, 10 w post Rx)
Alkhafajy et al. Iraq (2018)	CO_2 NR	90	No	25-55	21%	Vaginal relaxation ± UI	NR	Symptoms of loose vagina + ↓sexual gratification	Subjective 3 grade improvement scale for UI, vaginal tightening sensation (3 w post Rx)	Subjective 3 grade improvement scale for sexual gratification (3 w post Rx)
Anthanasiou et al. Greece (2019)	CO_2 SmartXide^2 V^{2}LR, Monalisa Touch, Deka, Florence, Italy	94	No	57 (min 44 - max 71)	100%	GSM ± UI	NR	Max POP-Q gr I	VAS for dyspareunia, dryness and itching/burning (Baseline, 1, 3, 6, 12 m post Rx)	ICIQ-FLUTS, ICIQ-UI SF, UDI-6, FSFI (Baseline, 1, 3, 6, 12 m post Rx)
Behnia-Willison et al. Australia (2017)	CO_2 SmartXide^2 V^{2}LR, Monalisa Touch, Deka, Florence, Italy	102	No	61 ± 7	100%	GSM ± UI ± POP	NR	NR	VHI, questionnaires for GSM symptoms (Baseline, 2 - 4, 12 - 24 m after 1st Rx)	APFQ (Baseline, 2 - 4, 12 - 24 m after 1st Rx)
Behnia-Willison et al. Australia (2019)	CO_2 SmartXide^2 V^{2}LR, Monalisa Touch, Deka, Florence, Italy	58	No	57.4 ± 11.4	77.6%	SUI	History of SUI	Max POP-Q gr I	APFQ (Baseline, 3, 12 - 24 m post Rx)	No secondary outcomes
Fistonić et al. Croatia (2015)	ER: YAG XS Dynamics, Fotona, Ljubljana, Slovenia	73	No	47 (IQR 41-54)	30.1%	SUI	NR	POP, if not "severe"	ICIQ-UI SF (Baseline, 1, 2 - 6 m post Rx)	US residual urine volume, Apimedis perineometer, PISQ-12 (Baseline, 1, 2 - 6 m post Rx)
Fistonić et al. Croatia (2016)	ER: YAG XS Dynamics, Fotona, Ljubljana, Slovenia	31	No	46.6 ± 9.1	NR	SUI	NR	POP, if not "severe"	ICIQ-UI SF (Baseline, 1, 2, 6 m post Rx)	US residual urine volume, Apimedis perineometer (Baseline, 1, 2, 6 m post Rx)
Fistonić et al. Croatia (2018)	ER: YAG XS Dynamics, Fotona, Ljubljana, Slovenia	84	No	48 (IQR 41-54)	31%	SUI	History of UI	POP-Q gr ≤I	ICIQ-UI SF (Baseline, 2 - 6 m post Rx)	Apimedis perineometer (Baseline, 2 - 6 m post Rx)
Gambacciani et al. Italy (2015a)	ER: YAG Fotona Smooth^{TM} XS, Fotona, Ljubljana, Slovenia	21	No	NR	100%	GSM ± SUI	NR	POP-Q gr ≤II	VAS (dryness and dyspareunia), VHI (Baseline, 4 w post Rx)	ICIQ-UI SF (Baseline, 4 w post Rx)

Gambacciani et al. Italy (2015b)	ER: YAG Fotona Smooth™ XS, Fotona, Ljubljana, Slovenia	19	No	NR	100%	GSM ± SUI	NR	POP-Q gr ≤II	VAS (dryness and dyspareunia) (Baseline, 4, 12, 24 w post Rx)	ICIQ-SF (Baseline, 4, 12, 24 w post Rx)
Gambacciani et al. Italy (2018)	ER: YAG Fotona Smooth™ XS, Fotona, Ljubljana, Slovenia	114	No	64.6 ± 4.4	100%	GSM ± SUI	NR	POP-Q gr <II	VAS (dryness and dyspareunia) (Baseline, 1, 3, 6, 12, 18, 24 w post Rx)	ICIQ-SF (Baseline, 1, 3, 6, 12, 18, 24 w post Rx)
Gaspar et al. Argentina (2017)	ER: YAG SP Spectro, Fotona, Ljubljana, Slovenia	22	No	57.9 (min 33-max 66)	NR	SUI	Urodynamics	NR	ICIQ-UI SF, 1-h pad weight test (Baseline, 3, 6 m after 1st Rx)	No secondary outcomes
Gaspar et al. Argentina (2018)	ER: YAG SP Spectro, Fotona, Ljubljana, Slovenia	29	No	66 (min 56-max 77)	100%	GSM + SUI	Urodynamics	POP-Q gr <II	ICIQ-UI SF, 1-h pad weight test (Baseline, 3, 6 m post Rx)	No secondary outcomes
González et al. Colombia (2018)	CO_2 SmartXide² V²LR, Monalisa Touch, Deka, Florence, Italy	161	No	53.38 ± 5.1	100%	GSM and SUI	NS	POP-Q gr <II	ICIQ-UI SF, 1-h pad weight test (Baseline, 12, 24, 36 m post Rx)	No secondary outcomes
Lin YH et al. Taiwan (2017)	ER: YAG XS Dynamics, Fotona, Ljubljana, Slovenia	30	No	52.6 ± 8.8	NR	SUI	Urodynamics	POP-Q gr ≤II	OABSS, ICIQ-UI SF, UDI-6, IIQ-7, POPDI-6, 1-h pad weight test, urodynamic testing, (Baseline, 1, 3, 12 m post Rx)	Perineometry, PISQ-12 (Baseline, 1, 3, 12 m post Rx)
Lin HY et al. Taiwan (2018)	Er: YAG & CO_2 Fotona SMOOTH™ SP, Fotona, Ljubljana, Slovenia SmartXide² V²LR, Monalisa Touch, Deka, Florence, Italy	31	No	48.4 ± 12.7	44.8%	SUI	History of UI	POP-Q gr ≤II	ICIQ-UI SF, 1-h pad weight test (Baseline, 2 m post Rx)	FSFI (Baseline, 2 m post Rx)
Lin KL et al Taiwan (2019)	ER: YAG Fotona Smooth™ XS, Fotona, Ljubljana, Slovenia	41	No	45.9 ± 7.2	31.7%	SUI	History of UI	NR	ICIQ-UI SF, UDI-6, IIQ-7 (Baseline, 6 m post Rx)	OABSS, Perineal US, vaginal pressure, POPDI-6 (Baseline, 6 m post Rx)
Mohajeri et al. Iran (2018)	CO_2 SmartXide² V²LR, Monalisa Touch, Deka, Florence, Italy	31	No	63.5 ± 9.5	100%	OAB	Urodynamics	POP-Q gr ≤II	ICIQ-OAB (Baseline, every month during Rx)	UDI-6, PISQ-12 (Baseline, every month during Rx)

Mothes et al. Germany (2018a)	ER: YAG MCL 31 Dermablate, ALT, Jena, Germany	16	No	71 ± 7	100%	Breast cancer survivors & recent POP surgery, and GSM ± UI ± vaginal laxity	History grade I UI	POP-Q gr <II No vaginal laxity	VHI, Vaginal pH (6 w post Rx)	Subjective judgment (6 w post Rx)
Mothes et al. Germany (2018b)	ER: YAG MCL 31 Dermablate, ALT, Jena, Germany	71	No	60.65 ± 12.57	79%	≥1 symptom out of SUI/ POP/GSM/ vaginal laxity/OAB	NR	POP-Q gr <II	VHI, Vaginal pH (6 w post Rx)	Subjective satisfaction (6 w post Rx)
Ogrinc et. al. Slovenia (2015)	ER: YAG SP Spectro, Fotona, Ljubljana, Slovenia	175	No	49.7 ± 10	NR	SUI or MUI	History of UI	NR	ICIQ-UI SF, ISI (Baseline, 2, 6, 12 m post Rx)	Subjective satisfaction, clinical examination, US, perineometry (Baseline, 2, 6, 12 m post Rx)
Ogrinc et. al. Slovenia (2017)	ER: YAG SP Spectro, Fotona, Ljubljana, Slovenia	61	No	54.9 ± 9.1	NR	POP	NR	Baden-Walker cystocele gr ≥II	Baden-Walker scale (Baseline, 2, 6, 12 post Rx)	Subjective satisfaction (2, 6, 12 m post Rx)
Pagano et al. Italy (2017)	CO_2 FemiLift CO_2 laser, Alma laser, Caesarea, Israel	33	No	52.3 ± 9.9	100%	GSM ± SUI	NR	POP-Q gr <II	VHI, VAS score dyspareunia (Baseline, 3 m post Rx)	ICIQ-UI SF (Baseline, 3 m post Rx)
Pardo et al. Chile (2016)	ER: YAG Fotona SmoothTM XS, Fotona, Ljubljana, Slovenia	42	No	46.5 (IQR 17)	NR	SUI	History of SUI	NR	ICIQ-UI SF (Baseline, 3 - 6 m post Rx)	Self-reporting of sexual gratification (Baseline, 3 - 6 m post Rx)
Perino et al. Italy (2016)	CO_2 SmartXide^2 V^{2}LR, Deka, Florence, Italy	30	No	56 (IQR 8.5)	100%	GSM + OAB	History of OAB	POP-Q gr <II	VHI, VAS (dryness, dyspareunia and burning/itching) (Baseline, 30 d post Rx)	Micturition diary, Patient`s Perception of Intensity of Urgency scale, OAB-Q SF (Baseline, 30 d post Rx)
Pitsouni et al. Greece (2016)	CO_2 SmartXide^2 V^{2}LR, Monalisa Touch, Deka, Florence, Italy	53	No	57.2 ± 5.4	100%	GSM	NR	POP-Q gr <II	VHI, VMV (Baseline, 4 w post Rx)	ICIQ-FLUTS, ICIQ-UI SF, UDI-6, KHQ, PGI-I, FSFI (Baseline, 4 w post Rx)

Samuels et al. USA (2019)	CO_2 CO_2RE Intima, Syneron Candela, Wayland, Massachusetts, USA	40	No	56 ± 8	100%	GSM ± UI	NR	POP-Q gr <II	VHI, vaginal biopsy (Baseline, 1, 3, 6, 12 m post Rx)	ICIQ-UI SF and questionnaire for satisfaction, FSFI and NSR dyspareunia (Baseline, 1, 3, 6, 12 m post Rx)
Tien et al. Taiwan (2017)	ER: YAG XS Dynamics, Fotona, Ljubljana, Slovenia	35	No	43.3 ± 7.2	20%	SUI	Urodynamics	NR	Bladder diary, urodynamics, UDI-6, 20-min pad test, KHQ, PPBC, USS, OABSS and IIQ-7 (Baseline, 3, 6 m post Rx)	FSFI, Modified male sexual activity questionnaire (Baseline, 3, 6 m post Rx)

Table S3. Laser settings and protocol ER: YAG laser.
Legend: Post Rx means after last therapy session Abbreviations: #, Number; µs, Microsecond; CO2, Carbon dioxide laser; d, Days; Er: YAG, Erbium-doped yttrium aluminium garnet laser; g, gram; Hz, Hertz; J, Joule; J/cm^2, Joule per square centimeters; J/Shot, Joule per Shot; m, Months; min, Minutes; mJ; Milli Joule; mJ/ppxl, Milli Joule per pixel; mm, Millimeter; ms, Millisecond; nm, Nanometer; PH, Phase; Rx, Treatment; SUI, Stress urinary incontinence; w, Week; W, Watt.

First author, Country (date)	Laser type & Brand	LASER SETTINGS					PROTOCOL					
		Pulse width	Spot size	Energy	Freq-uency	Dwell time	Pulse mode	Location	Average duration	# Treat-ment sessions	Time in-between	Follow-up
Alfarra et al. Saudi Arabia (2018)	ER: YAG Action II petite lady, Lutronic, Goyang, South Korea	250 ms and 1000 ms	NR	1,7 J/shot and 3,7 J/shot	NR	NR	Multiple micropuls e + long-pulsed mode	Vaginal wall	25 min	4	2 w and 1 m between 3th and 4th Rx	1, 2 m post Rx
Blaganje et al. Slovenia (2018)	ER: YAG XS Dynamics, Fotona, Ljubljana, Slovenia	NR	7 mm	10 J/cm^2	NR	NR	SMOOTH pulses	Vaginal wall, vestibulum + introitus	20 min	1	NR	3 m post Rx
Fistonić et al. Croatia (2015)	ER: YAG XS Dynamics, Fotona, Ljubljana, Slovenia	NR	NR	NR	NR	NR	SMOOTH mode	Vaginal wall and vestibulum	10 min	1	NR	1, 2 - 6 m post Rx
Fistonić et al. Croatia (2016)	ER: YAG XS Dynamics, Fotona, Ljubljana, Slovenia	250 ms	7 mm	3, 6 and 10 J/cm^2	1.6 Hz	NR	SMOOTH mode	Vaginal wall and vestibulum	10 min	1	NR	1, 2, 6 m post Rx
Fistonić et al. Croatia (2018)	ER: YAG XS Dynamics, Fotona, Ljubljana, Slovenia	NR	7 mm	10 J/cm^2	1.6 Hz	NR	SMOOTH mode	Vaginal wall and vestibulum	10 min	3	30 d	2 - 6 m post Rx
Gambacciani et al. Italy (2015a)	ER: YAG Fotona Smooth^{TM} XS, Fotona, Ljubljana, Slovenia	NR	7 mm	8.5 J and 3 J	NR	NR	SMOOTH mode	Vaginal wall, vestibulum and introitus	NR	3	30 d	4 w post Rx

Gambacciani et al. Italy (2015b)	ER: YAG Fotona Smooth™ XS, Fotona, Ljubljana, Slovenia	NR	7mm	5,5 J/cm^2 and 10 J/cm^2	1.6 Hz	NR	SMOOTH mode	Vaginal wall, vestibulum + introitus	NR	3	30 d	4, 12, 24 w post Rx
Gambacciani et al. Italy (2018)	ER: YAG Fotona Smooth™ XS, Fotona, Ljubljana, Slovenia	NR	7mm	6.0 J/cm^2	1.6 Hz	NR	SMOOTH mode	Vaginal wall, vestibulum + introitus	NR	3	30 d	1, 3, 6, 12, 18, 24 w post Rx
Gaspar et al. Argentina (2017)	ER: YAG SP Spectro, Fotona, Ljubljana, Slovenia	NR	2mm	6.0 J/cm^2	1.4Hz	NR	SMOOTH mode	Urethra	15 min	2	3 w	3, 6 m after 1st Rx
Gaspar et al. Argentina (2018)	ER: YAG SP Spectro, Fotona, Ljubljana, Slovenia	NR	4mm	1,5 J/cm^2	1.4Hz	NR	SMOOTH mode	Urethra	NR	2	3 w	3, 6 m post Rx
Lin YH et al. Taiwan (2017)	ER: YAG XS Dynamics, Fotona, Ljubljana, Slovenia	NR	NR	NR	NR	NR	SMOOTH mode	Vaginal wall, vestibulum + introitus	NR	2	4 w	1, 3, 12 m post Rx
Lin HY et al. Taiwan (2018)	Er: YAG (&CO_2) Fotona SMOOTH™ SP, Fotona, Ljubljana, Slovenia	NR	NR	3, 6 and 10 J/cm^2	NR	NR	Smooth mode	Vaginal wall and introitus	NR	1	NR	2 m post Rx
Lin KL et al Taiwan (2019)	ER: YAG Fotona Smooth™ XS, Fotona, Ljubljana, Slovenia	NR	NR	10 J/cm^2	1,6 Hz	NR	SMOOTH mode	Vaginal wall, vestibulum + introitus	NR	3	4 w	6 m post Rx
Mothes et al. Germany (2018a)	ER: YAG MCL 31 Dermablate, ALT, Jena, Germany	NR	NR	1st PH 15-35 J/cm^2 2nd PH 3-9 J/cm^2	NR	1st PH 300µs 2nd PH 1000µs	Pulse mode	Vaginal wall	10 min	1	NR	6 w post Rx
Mothes et al. Germany (2018b)	ER: YAG MCL 31 Dermablate, ALT, Jena, Germany	NR	NR	1st PH 15-35 J/cm^2 2nd PH 3-12 J/cm^2	NR	1st PH 300µs 2nd PH 1000µs	Pulse mode	Vaginal wall	10 min	1	NR	6 w post Rx
Ogrinc et. al. Slovenia (2015)	ER: YAG SP Spectro, Fotona, Ljubljana, Slovenia	250 ms	7mm	10 J/cm^2	1.6 Hz	NR	SMOOTH mode	Vaginal wall, vestibulum and introitus	NR	1 to 3	4 - 6 w for 2nd Rx 6 m for 3rd Rx	2, 6, 12 m post Rx
Ogrinc et. al. Slovenia (2017)	ER: YAG SP Spectro, Fotona, Ljubljana, Slovenia	NR	NR	NR	NR	NR	SMOOTH mode	Vaginal wall, vestibulum	15 min	2 to 5	2 m	2, 6, 12 m post Rx

Okui et al. Japan (2018)	ER: YAG Fotona Smooth™ XS, Fotona, Ljubljana, Slovenia	NR	NR	NR	NR	NR	SMOOTH mode	Vaginal wall, vestibulum	20 min	3	1 m	12 m post Rx
Okui et al. Japan (2019)	ER: YAG Fotona Smooth™ XS, Fotona, Ljubljana, Slovenia	NR	NR	NR	NR	NR	Long pulse setting	Vaginal wall	20 min	3	1 m	12 m after 1st Rx
Pardo et al. Chile (2016)	ER: YAG Fotona SmoothTM XS, Fotona, Ljubljana, Slovenia	NR	NR	3, 6 and 10 J/cm^2	1.6 Hz	NR	SMOOTH mode	Vaginal wall, vestibulum and introitus	NR	2	21 - 28 d	3 - 6 m post Rx
Tien et al. Taiwan (2017)	ER: YAG XS Dynamics, Fotona, Ljubljana, Slovenia	NR	NR	NR	NR	NR	SMOOTH mode	Vaginal wall and introitus	NR	1	NR	3, 6 m post Rx

Table S4. Laser settings and protocol CO2 laser.
Legend: Post Rx means after last therapy session Abbreviations: #, Number; µm, Micrometer; µs, Microsecond; CO2, Carbon dioxide laser; d, Days; Er: YAG, Erbium-doped yttrium aluminium garnet laser; g, gram; Hz, Hertz; J, Joule; J/cm2, Joule per square centimeters; J/Shot, Joule per Shot; m, Months; min, Minutes; mJ; Milli Joule; mJ/ppxl, Milli Joule per pixel; nm, Nanometer; PH, Phase; Rx, Treatment; SUI, Stress urinary incontinence; w, Week; W, Watt.

First author, Country (date)	Laser type & Brand	LASER SETTINGS Energy	Freq-uency	Dwell time	DOT spacing	Smart-Stak para-meter	PROTOCOL Pulse mode	Location	Average duration	# Treat-ment sessions	Time in-between	Follow-up
Alkhafajy et al. Iraq (2018)	CO_2 NR	NR	NR	NR	NR	NR	NR	Vaginal wall	NR	3	21 d	3 w post Rx
Anthanasiou et al. Greece (2019)	CO_2 SmartXide^2 V^{2}LR, Monalisa Touch, Deka, Florence, Italy	30 W and 40 W (24 W for introitus)	NR	1000 µs	1000 µm	1 to 3	D-pulse mode	Vaginal wall and introitus	NR	3 to 5	30 d	1, 3, 6, 12 m post Rx
Behnia-Willison et al. Australia (2017)	CO_2 SmartXide^2 V^{2}LR, Monalisa Touch, Deka, Florence, Italy	30 W and (20 W vestibulum)	NR	1000 µs	1000 µm	2 (1 for vestib-ulum)	D-pulse mode	Vaginal wall, vestibulum and fourchette	NR	3	6 w or more	2 - 4, 12 - 24 m after 1st Rx

Behnia-Willison et al. Australia (2019)	CO_2 SmartXide² V²LR, Monalisa Touch, Deka, Florence, Italy	40 W	NR	1000 µs	700 µm	3	D-pulse mode	Vaginal wall and external urethral meatus	NR	3	4 - 6 w	3, 12 - 24 m post Rx
González et al. Colombia (2018)	CO_2 SmartXide² V²LR, Monalisa Touch, Deka, Florence, Italy	NR	NR	NR	NR	NR	NR	Urethro-vesical junction	NR	4	40 - 45 d + at 12, 24, 36 m	12, 24, 36 m post Rx
Lin HY et al. Taiwan (2018)	CO_2 (& Er: YAG) SmartXide² V²LR, Monalisa Touch, Deka, Florence, Italy	30 and 20 W	NR	NR	NR	NR	NR	Vaginal wall and introitus	NR	1	NR	2 m post Rx
Mohajeri et al. Iran (2018)	CO_2 SmartXide² V²LR, Monalisa Touch, Deka, Florence, Italy	40 W and 24 W	NR	1000 µs	1000 µm	1-3	NR	NR	NR	3	1 m	Every month during Rx
Pagano et al. Italy (2017)	CO_2 FemiLift CO_2 laser, Alma laser, Caesarea, Israel	30 W 60-100 mJ/ppxl	0.5 Hz	NR	NR	NR	High laser mode	Vaginal wall	NR	3	1 m	3 m post Rx
Perino et al. Italy (2016)	CO_2 SmartXide² V²LR, Deka, Florence, Italy	40 W and (30 or 20 W for introitus)	NR	1000 µs	1000 µm	2	D-pulse mode	Vaginal wall and introitus	NR	3	30 d	30 d post Rx
Pitsouni et al. Greece (2016)	CO_2 SmartXide² V²LR, Monalisa Touch, Deka, Florence, Italy	30 or 40 W (24 W for introitus)	NR	1000 µs	1000 µm	1 to 3	D-pulse mode	Vaginal wall, introitus	NR	3	30 d	4 w post Rx
Samuels et al. USA (2019)	CO_2 CO_2RE Intima, Syneron Candela, Wayland, Massachusetts, USA	50-60 mJ; introitus: 45-60 mJ	NR	NR	NR	NR	Deep mode	Vaginal wall and introitus	Vagina: 6 ± 3 min Introitus: 3 ± 2 min	3	30 d	1, 3, 6, 12 m post Rx

Table S5. Results according to the initial diagnosis on entry into the study.
Legend: Significance is displayed for outcome measures in the second last column, in the order of appearance. Abbreviations: % of pt: percentage of patients; ↑, improvement; ↓, reduction; 1-h pad test, one hour pad test; APFQ, Australian Pelvic Floor Questionnaire; CO2, Carbon dioxide laser; d, Day; DM, Diabetes mellitus; Er: YAG, Erbium-doped yttrium aluminium garnet laser; FSFI, Female Sexual Function Index; gr, Grade; GSM, Genitourinary syndrome of menopause; ICIQ-FLUTS, Questionnaire for evaluating female lower urinary tract symptoms and impact on quality of life; ICIQ-UI SF, International Consultation on Incontinence Questionnaire - Urinary Incontince short form; ICS, International Continence Society; IIQ-7, Short form of the Incontinence Impact Questionnaire; IQR, Interquartile range; ISI, International severity index; KHQ , Kings` health questionnaire; min, Minimum; max, Maximum; m, Month; MUI, Mixed urinary incontinence; NR, Not reported; NSR, 10-point numerical scale response; OAB, Overactive bladder; OABSS, Overactive Bladder Symptom Score; OAB-Q SF, Overactive Bladder Questionnaire - short form; PGI-I, Patients Global Impression of Improvement; pH, Power of hydrogen; PISQ-12, Short form of the Pelvic Organ Prolapse/Urinary

Incontinence Sexual Questionnaire; POP, Pelvic Organ Prolapse; POPDI-6, Pelvic Organ Prolapse Distress Inventory; POP-Q, Pelvic Organ Prolapse Quantification; PPBC, Patient perception of Bladder condition; pt, Patient; RCT, Randomized controlled trail; Rx, Treatment; SUI, Stress urinary incontince; TOT, Transobturator tape; TVT, Tension free vaginal tape; UDI-6, Urogenital Distress inventory; UI, Urinary incontinence; US, Ultrasound; USS, Urgency severity scale; UTI, Urinary tract infection; UUI, Urge urinary incontinence; VAS, Visual analogue scale; VHI, Vaginal health index; VMV, Vaginal maturation value; w, Week; y, Year.

First Author (date)	Laser	Outcome measures: Timepoints	Outcome measures: Tool	Results	Significance
			SUI		
Alfarra et al. (2018)	ER: YAG	Baseline, 1, 2 m post Rx FSFI: 10 w post Rx	Bladder diary PFX2 FSFI	"significant" ↓ symptoms of UI "significant" ↑ of vaginal muscle power in all patients 2 m post Rx 57% pt very satisfied and 42.3% moderately satisfied for sexual satisfaction	NR NR NR
Behnia-Willison et al. (2019)	CO_2	Baseline, 3, 12 - 24 m post Rx	APFQ	82% pt ↓ in symptoms of SUI (to mild or no SUI) 3 m post Rx 71% pt ongoing improvement at 12-24 m post Rx All bladder function measures significantly improved	<0.01 <0.01 ≤0.01
Blaganje et al. (2018)	ER: YAG	Baseline, 3 m post Rx	ICIQ-UI SF, PISQ-12, FSFI Myomed 623 perineometer	ICIQ-UI SF, PISQ-12 and FSFI more improved in laser group than sham 21% of patients in laser were dry versus 4% in sham group Duration and max pressure ↑ compared to sham	<0.001; 0.014; 0.025 0.006 0.05
Fistonić et al. (2015)	ER: YAG	Baseline, 1, 2 - 6 m post Rx	ICIQ-UI SF	ICIQ-UI SF ↓ to a median of 46% Higher reduction in pt with normal BMI and <39 y ↔ overweight pt and >60 y	<0.001 <0.001
Fistonić et al. (2016)	ER: YAG	Baseline, 1, 2, 6 m post Rx	ICIQ-UI SF US residual urine volume Apimedis perineometer	ICIQ-UI SF ↓ at 1, 2 and 6 m post Rx compared to baseline 32.5% pt score 0 on ICIQ-UI after 1 m ↓ post void residual urine volume in 1m and 2m No sig improvement in perineometry measurements	<0.001; 0.005; 0.01 NR 0.01; 0.04 ≥ 0.09
Fistonić et al. (2018)	ER: YAG	Baseline, 2 - 6 m post Rx	ICIQ-UI SF	Age and pre-Rx ICIQ-UI SF values → independent predictors Rx efficacy for SUI ↓ ≥ 30% on ICIQ-UI associated with BMI and ICIQ-UI values before Rx All pt with 4-5 positive predictors had a ↓ ≥ 30% on ICIQ-UI	0.014; <0.001 0.002 NR
Gaspar et al. (2017)	ER: YAG	Baseline, 3, 6 m after 1st Rx	ICIQ-UI SF 1-h pad weight test	Cured pt: 64% at 3 m, 46% at 6 m, Improved pt: 18 % at 3 m and 23% at 6 m 1-h pad test ↓ with (≥ 50% of pad weight by 82% pt at 3 m and 50% pt at 6 m	<0.001 <0.001
Lin YH et al. (2017)	ER: YAG	Baseline, 1, 3, 12 m post Rx	1-h pad weight test OABSS, ICIQ-UI SF, UDI-6, POPDI-6 IIQ-7, PISQ-12 perineometry,	3m post Rx: 1-h pad test ↓ from 13.2 ± 17.7 g to 6.1 ± 11.6 g 3m post Rx: ↓ in OAB symptoms in 4 questionnaires ↓ in ICIQ-UI SF and POPDI-6 also at 12m post Rx No sig improvements in IIQ-7 and PISQ-12 vaginal pressure ↑ 62.5% pt and 54.2% partners reported ↑ sexual gratification	0.039 ≤ 0.027 0.001 ≥0.227 0.009 NR
Lin HY et al. (2018)	Er: YAG & CO_2	Baseline, 2 m post Rx	ICIQ-UI SF 1-h pad weight test, FSFI	Er: YAG group: ICIQ-UI SF ↓ from 8.25 to 5 CO_2 laser group: ICIQ-UI SF ↓ from 11.11 to 6.44 1-h pad weight test: no sig difference, FSFI no sig difference	0.007 0.035 0.224; 0.389
Lin KL et al. (2019)	ER: YAG	Baseline, 6 m post Rx	ICIQ-UI SF UDI-6, IIQ-7, OABSS, POPDI-6, Perineal US vaginal pressure	↓ on median ICIQ-UI SF from 7.2 ± 4.5 to 3.7 ± 3.5 at 6 m post-Rx Improvement in all 4 questionnaires 6 m post Rx Bladder neck mobility by perineal US ↓	<0.001 ≤ 0.001 0.039 ≤ 0.136 NR

				No sig improvement in vaginal pressure Rx efficacy for SUI at 6 m post-Rx was 75.5%	
Mothes et al. (2018b)	ER: YAG	6 w post Rx	Subjective judgment	82% (n=58) positive overall judgment of the treatment	NR
Ogrinc et. al. (2015)	ER: YAG	Baseline, 2, 6, 12 m post Rx	ISI ICIQ-UI SF	↓ ISI: baseline Mild UI: ↓ 2.6 ± 1.0 ↔ baseline Moderate UI: ↓ 3.6 ± 1.4 ↔ baseline Severe UI: ↓ 5.7 ± 1.8 ↔ baseline Very severe UI: ↓ 8.4 ± 2.6 77% pt with SUI ↔ 34% with MUI had no UI after 12 m, no influence of age	<0.001 <0.001 <0.001
Okui et al. (2018)	ER: YAG	Baseline, 12 m post Rx	1-h pad test, ICIQ-UI SF	≈ improvements in SUI for all 3 groups (1-h pad test and ICIQ-UI SF)	<0.001; <0.001
Pardo et al. (2016)	ER: YAG	Baseline, 3 - 6 m post Rx	ICIQ-UI SF self-reporting of sexual gratification	↓ on median ICIQ-UI SF from 11 to 3 at 6 m follow-up 81.8% pt reported improvement of sexual gratification 78,6% pt reported improvement and 38.1% pt complete healing of SUI	<0.001 NR NR
Tien et al. (2017)	ER: YAG	Baseline, 3, 6 m post Rx	20-min pad test PPBC, USS, OABSS, UDI-6, IIQ-7, KHQ, FSFI, Urodynamics Modified male sexual activity questionnaire	Mild SUI at baseline: cured (≤1 g) 50%, improved (↓ ≥ 50% pad weight) 27.8% ↔ Moderate to severe SUI at baseline: cured 20%, improved 60% at 6 m ↑ on all questionnaires, ↑ on 10/13 items of KHQ and on desire domain FSFI Urodynamic functions did not differ across the timeline Sexual activity ↑ in 53.5% of male partners at 3 m, 40% at 6 m post Rx	NR NR <0.05; <0.05; 0.02 NR NR
				OAB	
Mohajeri et al. (2018)	CO_2	Baseline, every month during Rx	ICIQ-OAB UDI-6 PISQ-12	↓ in ICIQ-OAB score and urgency during Rx sessions ↓ in UDI-6 score, but only after 3 Rx sessions No improvements in nocturia, frequency, leakage and PISQ-12 score	<0.05; ≤0.001 <0.05 ≥0.05
Okui et al. (2019)	ER: YAG	Baseline, 12 m after 1st Rx	OABSS	↓ on all items of OABSS in all 3 groups → Laser ≈ efficacy to 4 mg Fesoterodine and 25 mg Mirabegron	<0.001
				POP	
Alkhafajy et al. (2018)	CO_2	3 w post Rx	Subjective 3 gr scale for UI, vaginal tightening sensation and sexual gratification	↑ vaginal tightening and sexual functions after Rx ↓ in light bladder leak and vaginal dryness & recurrent infection after Rx	0.006; 0.004 0.01; 0.02
Ogrinc et. al. (2017)	ER: YAG	Baseline, 2, 6, 12 m post Rx	Baden-Walker scale	Improvement in BW grade after 1st Rx, POP ↓ by at least one BW gr in 95% pt, with 85% pt presenting with BW gr 0 or I, remaining 15% with BW gr II 57% pt very satisfied and 33% pt satisfied with Rx	<0.001
				GSM	
Anthanasiou et al. (2019)	CO_2	Baseline, 1, 3, 6, 12 m post Rx	ICIQ-FLUTS, ICIQ-UI SF, UDI-6, FSFI	↓ in all outcomes from baseline to 1st m, maintaining all 12 m follow-up At 1 and 12m post Rx, sig difference on outcome measures between 3 and 4 and between 4 and 5 Rx sessions, not between 4 and 5 sessions	All <0.001; all <0.001 <0.05 <0.05; NR
Behnia-Willison et al. (2017)	CO_2	Baseline, 2 - 4, 12 - 24 m post Rx	APFQ	Improvement of sexual function, POP, bladder function and UUI	≤0.005; 0.001; 0.001; 0.003

Gambacciani et al. (2015a)	ER: YAG	Baseline, 4 w post Rx	ICIQ-UI SF	↓ in ICIQ-UI SF scores after Rx Scores remained sig lower than basal values at 4 w follow-up	<0.01 <0.01
Gambacciani et al. (2015b)	ER: YAG	Baseline, 4, 12, 24 w post Rx	ICIQ-UI SF	↓ in ICIQ-UI SF scores after Rx The scores remained sig lower than basal values at 4 , 12 and 24 w follow-up	<0.01 <0.01; <0.01; <0.01; <0.01
Gambacciani et al. (2018)	ER: YAG	Baseline, 1, 3, 6, 12, 18, 24 w post Rx	ICIQ-UI SF	↓ in the ICIQ-UI SF scores after Rx The scores remain sig lower than basal values at1, 3, 6 and 12 m follow- up The values at 18 and 24 m where not sig different from baseline	<0.05 <0.01 NR
Gaspar et al. (2018)	ER: YAG	Baseline, 3, 6 m post Rx	ICIQ-UI SF 1-h pad weight test	↓ in ICIQ-SF scores and improvement on 1-h pad test ↓ dysuria, frequency and urgency ate 3 and, 6 m follow-up	<0.0005; <0.0005 <0.0005; <0.0005; <0.0005
González et al. (2018)	CO_2	Baseline, 12, 24, 36 m post Rx	ICIQ-UI SF, 1-h pad weight test	↓ in ICIQ-UI SF scores and 1-h pad weight test at 12, 24 and 36 m follow-up	<0.001; <0.001; <0.001
Mothes et al. (2018a)	ER: YAG	6 w post Rx	Subjective judgment	94% (n=15) positive overall judgment of the treatment	NR
Pagano et al. (2017)	CO_2	Baseline, 3 m post Rx	ICIQ-UI SF	↓ ICIQ-UI scores at 3 follow-up 90% pt were satisfied	<0.01 NR
Perino et al. (2016)	CO_2	Baseline, 30 d post Rx	Micturition diary, Patient`s Perception of Intensity of Urgency scale, OAB-Q SF	Improvement in micturition diary, ↓ # urge episodes and OAB-Q SF scores	<0.0001; <0.0001; <0.0001;
Pitsouni et al. (2016)	CO_2	Baseline, 4 w post Rx	ICIQ-FLUTS, UDI-6, ICIQ-UI SF, KHQ FSFI	↓ ICIQ-FLUTS, UDI-6, ICIQ-UI SF and KHQ scores Improvement in FSFI score	<0.001; <0.001; <0.001; <0.001 <0.001
Samuels et al. (2019)	CO_2	Baseline, 1, 3, 6, 12 m post Rx	ICIQ-UI S FSFI	↓ frequency of leaking: baseline/after 1st Rx/6 m/12 m -62.5%/ 52%/72%/64% Improvement in FSFI after 1st Rx in 88% pt, at 12 m follow-up in 83% Satisfaction rate 68% at 6 m, 75% at 12 m.	NR <0.001; NR <0.05

Appendix S1. Search strategy.

Concept 1: Urinary incontinence

"Urinary Incontinence"[Mesh] OR "urinary incontinence"[tiab] OR incontinence[tiab] OR "incontinentia urinae"[tiab] OR "urinary leakage"[tiab] OR "urinary leakage"[tiab].

Concept 2: Pelvic Organ Prolapse

"Pelvic Organ Prolapse"[Mesh] OR pelvic-organ-prolapse*[tiab] OR urogenital-prolapse*[tiab] OR vaginal-vault-prolapse*[tiab] OR cystocele[tiab] OR cystocoele[tiab] OR "urinary bladder prolapse"[tiab] OR rectal-prolapse*[tiab] OR anus-prolapse*[tiab] OR uterine-prolapse*[tiab] OR vaginal-prolapse*[tiab] OR "genital prolapse"[tiab] OR "genito-urinary prolapse"[tiab] OR "genitourinary prolapse"[tiab] OR "pelvic descent"[tiab] OR "pelvic organ descent"[tiab] OR "pelvic prolapse"[tiab] OR "vaginal descensus"[tiab] OR "vaginal descent"[tiab] OR "vaginal wall prolapse"[tiab]

Concept 3: Laser therapy

"Lasers"[Mesh:NoExp] OR laser*[tiab] OR "Laser therapy"[MeSH Terms] OR "laser therapy"[tiab] OR "laser therapies"[tiab] OR pulsed-laser*[tiab] OR "Lasers, Solid-State"[Mesh] OR Solid-State-Laser*[tiab] OR Er-YAG[tiab] OR "Yttrium Aluminum Garnet"[tiab] OR Erbium-YAG[tiab] OR YAG[tiab] OR "Lasers, gas"[MeSH Terms] OR CO2-laser*[tiab]

The three concepts were composed as following: (concept 1 OR concept 2) AND concept 3.

Appendix S2. Risk-of-bias assessment.

ROBINS-I TOOL

Legend: low risk of bias ●, moderate risk of bias ●, serious risk of bias ●, critical risk of bias ●, no information ●; The Cochrane collaboration tool: low risk of bias ●, high risk of bias ●, unclear risk of bias ●.

	ROBINS-I TOOL							
STUDIES	**DOMAIN**							
	Pre-intervention		***At inter-vention***	***Post-intervention***				***Overall***
	Confounding	**Selection of participants**	**Classification of interventions**	**deviations from intended ...**	**Missing data**	**Measurement of outcomes**	**Selection of the reported result**	
Fistonić et al. (2015)	●	●	●	●	●	●	●	●
Fistonić et al. (2016)	●	●	●	●	●	●	●	●
Fistonić et al. (2018)	●	●	●	●	●	●	●	●
Gaspar et al. (2018)	●	●	●	●	●	●	●	●
Mohajeri et al. (2018)	●	●	●	●	●	●	●	●
Pitsouni et al. (2016)	●	●	●	●	●	●	●	●
Alfarra et al. (2018)	●	●	●	●	●	●	●	●
Anthanasiou et al. (2019)	●	●	●	●	●	●	●	●
Behnia-Willison et al. (2017)	●	●	●	●	●	●	●	●
Behnia-Willison et al. (2019)	●	●	●	●	●	●	●	●
Gambacciani et al. (2015a)	●	●	●	●	●	●	●	●
Gambacciani et al. (2015b)	●	●	●	●	●	●	●	●
Gambacciani et al. (2018)	●	●	●	●	●	●	●	●
González et al. (2018)	●	●	●	●	●	●	●	●
Lin YH et al. (2017)	●	●	●	●	●	●	●	●
Ogrinc et. al. (2015)	●	●	●	●	●	●	●	●
Ogrinc et. al. (2017)	●	●	●	●	●	●	●	●
Okui et al. (2018)	●	●	●	●	●	●	●	●
Okui et al. (2019)	●	●	●	●	●	●	●	●
Pagano et al. (2017)	●	●	●	●	●	●	●	●
Perino et al. (2016)	●	●	●	●	●	●	●	●
Samuels et al. (2019)	●	●	●	●	●	●	●	●
Tien et al. (2017)	●	●	●	●	●	●	●	●
Gaspar et al. (2017)	●	●	●	●	●	●	●	●
Lin KL et al (2019)	●	●	●	●	●	●	●	●
Mothes et al. (2018a)	●	●	●	●	●	●	●	●
Mothes et al. (2018b)	●	●	●	●	●	●	●	●
Pardo et al. (2016)	●	●	●	●	●	●	●	●
Alkhafajy et al. (2018)	●	●	●	●	●	●	●	●
Lin HY et al. (2018)	●	●	●	●	●	●	●	●

THE COCHRANE COLLABORATIONS'S TOOL

Legend: low risk of bias ●, moderate risk of bias ●, serious risk of bias ●, critical risk of bias ●, no information ●; The Cochrane collaboration tool: low risk of bias ●, high risk of bias ●, unclear risk of bias ●.

STUDIES	THE COCHRANE COLLABORATIONS'S TOOL DOMAIN						
	Selection bias		***Performance bias***	***Detection bias***	***Attrition bias***	***Reporting bias***	***Other bias***
	Random sequence generation	**Allocation concealment**	**Blinding of participants and personnel**	**Blinding of outcome assessment**	**Incomplete outcome data**	**Selective reporting**	**Other sources of bias**
Blaganje et al. (2018)	●	●	●	●	●	●	●

Chapter 6

Vaginal Er:YAG LASER application in the menopausal ewe model: a randomised oestrogen and sham controlled trial

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ABSTRACT:

Objective: To describe effects of non-ablative Er:YAG LASER on vaginal atrophy induced by iatrogenic menopause in the ewe.

Design: Animal experimental, randomised, sham and oestrogen-treatment controlled study with blinding for primary outcome.

Setting: KU Leuven, Belgium.

Sample: Twenty-four ewes

Methods: Menopause was surgically induced, after which the ewes were randomised to three groups receiving either (1) vaginal Er:YAG LASER application three times, with a one-month interval; (2) three sham manipulations with a month interval; or (3) oestrogen replacement and sham manipulations. At given intervals, ewes were clinically examined and vaginal wall biopsies taken. Vaginal compliance was determined by passive biomechanical testing from explants taken at obduction.

Main Outcome Measures: Vaginal epithelial thickness (primary), composition of the lamina propria (collagen, elastin, glycogen and vessels content), vaginal compliance, clinical signs.

Results: Animals exposed to Er:YAG LASER application and sham manipulation, but not to oestrogens, displayed a significant and comparable increase in vaginal epithelial thickness between baseline and seven days after the third application (69% and 67% respectively, both $p<.0008$). In LASER treated ewes, temporary vaginal discharge and limited thermal injury were observed. Oestrogen substituted ewes displayed a more prominent increase in epithelial thickness (202%; $p<.0001$) and higher vaginal compliance ($p<.05$). None of the interventions induced changes in the lamina propria.

Conclusions: Vaginal Er:YAG LASER has comparable effect to sham manipulation in menopausal ewes.

Funding: Fotona LASER (Ljubljana, Slovenia); Grant Progress Q34, Charles University, Prague, Czechia.

Keywords: LASER, sheep, atrophy, vagina, oestrogen, epithelium, planar biaxial testing

Tweetable abstract: Vaginal Er:YAG LASER has comparable effect to sham manipulation in menopausal ewes #LASER #GSM #RCT

INTRODUCTION:

Genitourinary syndrome of menopause (GSM) is a collection of signs and symptoms in the lower urogenital tract associated with low-oestrogen levels[25]. Main subjective complaints are genital burning, itching, irritation, lack of lubrication, sexual discomfort or pain, urinary symptoms of urgency, dysuria and recurrent urinary tract infections[25]. Objectively, low circulating oestrogen levels lead to thinning of the vaginal and uro-epithelium, an increase in vaginal pH, a decrease in collagen and tissue elasticity and fewer blood vessels[26].

Although vaginal atrophy may be present in up to 90% menopausal women, only 30% has subjective complaints[27]. GSM may also develop in younger women, when hormonal sensitive tumours are treated either with surgical oophorectomy or by anti-oestrogen treatment[28].

Mild GSM may be treated with a change in lifestyle[29, 30], lubricants[31] and herbal remedies[32, 33]. For mild to severe GSM, topical or systemic hormonal treatment has been shown to be effective[34]. However, not all women can or wish to use hormones[35].

Recently, non-ablative LASER therapy has been proposed as an alternative, using either erbium-doped yttrium aluminium garnet (Er:YAG) and carbon dioxide (CO_2). Both LASER sources are previously used in dermatology, e.g. for treating wrinkles by "skin rejuvenation"[38], which theoretically can also be applied vaginally[251]. It is believed that vaginal atrophy is reversed through activation of heat shock proteins, neoangiogenesis, neocollagenogenesis, an increase of epithelial thickness and lowering of the vaginal pH[36, 39-41, 252]. LASER treatment of GSM has become quickly embraced, though remains investigational[253]. In 2018, the FDA warned that the effectiveness and safety of vaginal "rejuvenation" was not established[211].

To fill the gap in evidence, we decided to perform a randomised controlled study to test effects of Er:YAG LASER in the sheep model of menopause. Our aim was to identify the effects of Er:YAG LASER on the vaginal wall compared to sham vaginal manipulation with and without systemic oestradiol administration.

Our primary outcome was the change in vaginal epithelial thickness. Secondary outcomes were changes to the lamina propria in terms of collagen, elastin and vessels contain, clinical effect on the epithelium and changes to the biomechanical behaviour of vaginal tissue.

METHODS:

Study design:

After cycle synchronization[254], 24 ewes underwent ovariectomy (OVX; day 0) to mimic menopause[255, 256], and were randomised to three treatment groups (Er:YAG-LASER, hormone replacement therapy (HRT) and sham). Animals were regularly clinically assessed throughout the experiment (Table S1). Assessors of the histologic specimens, used for measurement of the primary outcome measure, were blinded to the treatment group; blinding was not possible when manipulating the sheep. Treatment started 180 days after ovariectomy, and the experiment ended at 280 d.

Experimental procedures:

Er:YAG LASER:

A multiple use clear glass speculum (Mclear, Fotona, Ljubljana, Slovenia) was inserted vaginally, followed by a circular adaptor and a full-field collimated handpiece (ID 88920 resp. R11, Fotona, Ljubljana, Slovenia). Then, the LASER energy was applied (Figure 1 A) using a non-ablative SMOOTH IntimaLase protocol^{TM42} for the first two applications and the RenovaLase protocol^{TM42} (Table S2) for the third application. These protocols should provide

energy deposition in the lamina propria (200µm under the surface) across the full circumference of the entire vaginal canal[36]. After the second application we noticed local thermal injury in the vagina, so that we lowered the fluence of the LASER[42] (Figure 2 D, E). Vaginal manipulation did not require any anaesthesia because it is painless in nature.

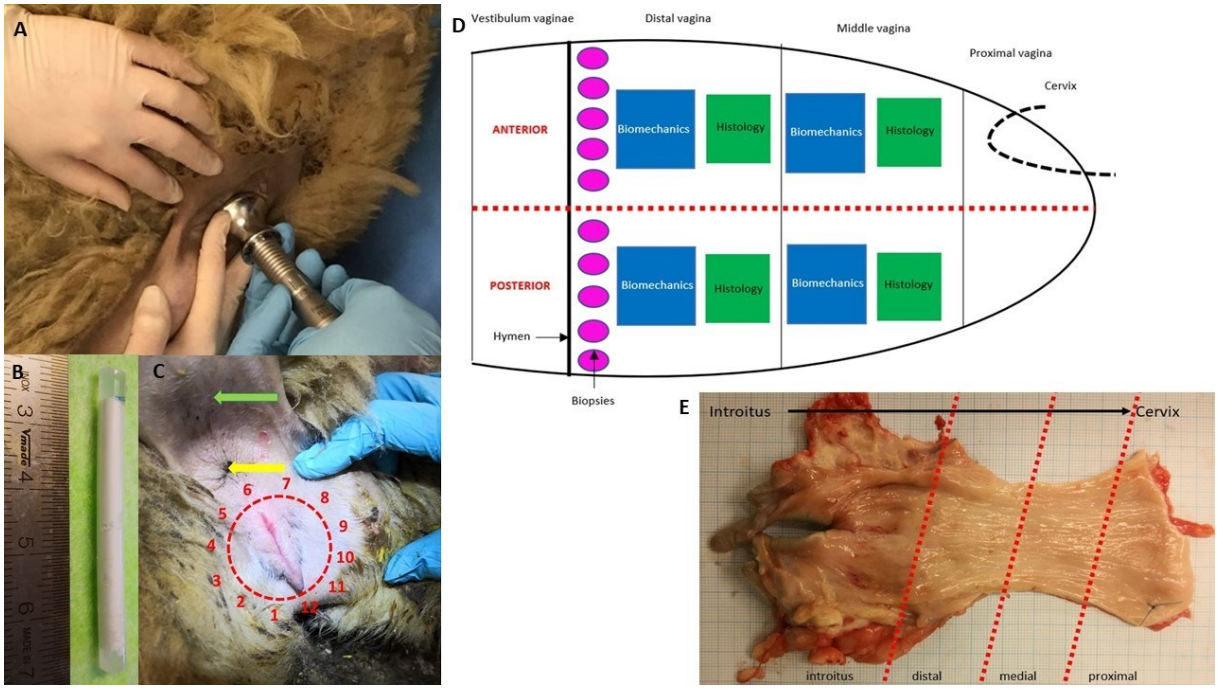

Figure 1. A: Application of Er:YAG LASER **B:** Photograph of an estradiol implant **C:** Schematic figure of locations where biopsies were taken. Arrows: green = tail, yellow = anus. **D:** Localization of biopsies and division of samples of explants **E:** Vaginal explant. Yellow circle represents location of urethral ostium.

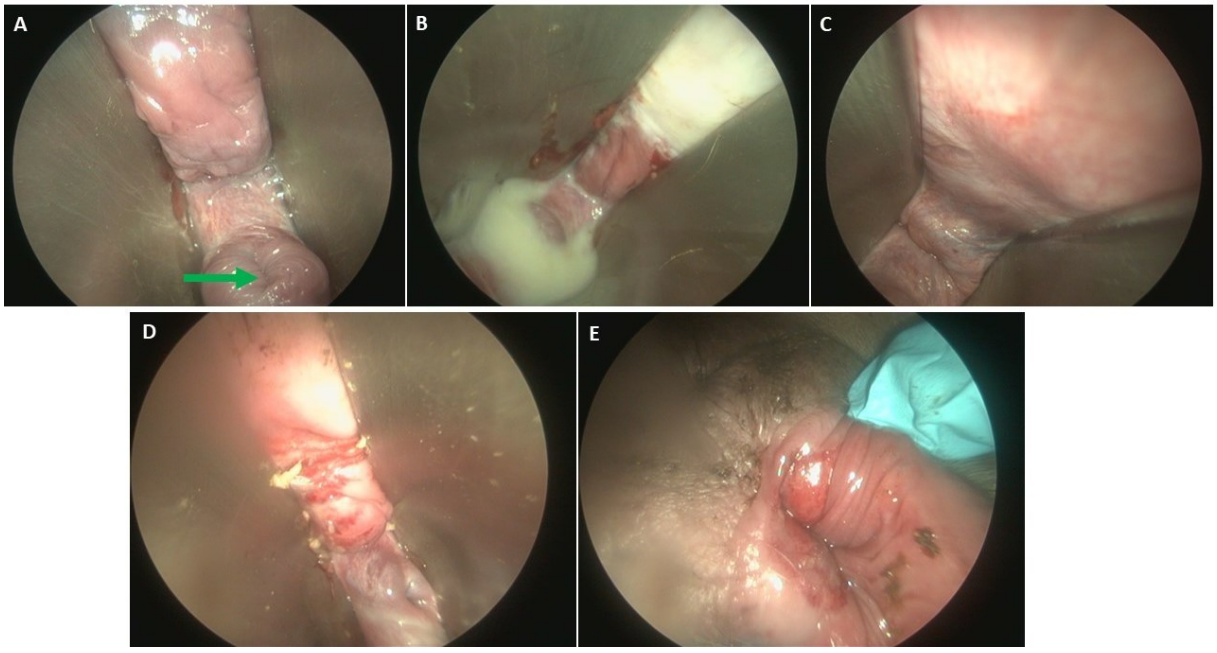

Figure 2. Top row: representative photographs during clinical examination, at 193d in **A:** oestrogen, **B:** LASER and **C:** sham ewes. The whitish discharge (B) was visible for one week. **Bottom row:** Thermal injury on **D:** the posterior vaginal wall at 223d and **E:** introitus. Green arrow – urethral ostium along the anterior vaginal wall.

Sham therapy:

Vaginal manipulation was done with the same glass speculum, and lasted approximately five minutes, which is comparable to the duration of the laser application in clinical practice. No LASER energy was applied.

Hormonal replacement therapy:

An oestradiol implant (Figure 1 B) in the form of sustained release system, was inserted under the skin in the inguinal region at the time of the first LASER application 180 days after ovariectomy (details in Appendix S1). In addition, those animals underwent sham vaginal manipulations as described above.

Cycle synchronization:

Epithelial thickness (ET) is changing cyclically in the ewes[257, 258], including the epithelial thickness (40-100 µm), with the highest value at oestrus and the lowest at proestrus and dioestrus[259]. Ewes were therefore first synchronized by insertion of a sponge with 60 mg medroxyprogesterone acetate (Veramix, Pfizer, IJssel, The Netherlands) for 14 days. Typically, after the sponge removal, oestrus is present in most ewes after 2-3 days[254].

Bilateral ovariectomy:

Ovariectomy was performed four days after synchronization (detailed in Appendix S2).

Core outcome set (COS):

No core outcome set was used, neither is there one available[260] for this condition.

Clinical inspection:

Clinical inspection was performed regularly with a gynaecological speculum to screen for any epithelial abnormalities: colour of the vaginal epithelium (erythema), petechiae, bleeding, inflammation, erosion, oedema, scar, amount of fluid. Findings were filled in on a display with vaginal health index (Vaginal health index scoring in Table S3) and images were taken by a portable endoscopic camera (Telepack X; Karl Storz; Tuttlingen, Germany). The vaginal pH was measured semi-quantitatively by pH strips (Sigma-Aldrich, Diegem, Belgium; range 7-14, resolution 0.5).

Biopsies:

The vagina was locally irrigated with sodium hypochlorite 0.5g/100mL (Melisana, Brussels, Belgium) and anesthetized with lidocaine hydrochloride gel (Xylocaine®, AstraZeneca, Dilbeek, Belgium). A biopsy (ø 5 mm) was taken. To avoid overlap, biopsies were taken consecutively in the clockwise direction (12 h at the urethral ostium) along a circular line one cm above the hymen (Figure 1 C, D) and fixed in paraformaldehyde 4%. In total, 11 biopsies were obtained during the experiment at the time points shown in Table S1.

Obduction:

Details are provided in Appendix S3. The vestibulum, vagina, uterus and urinary bladder were harvested en bloc with surrounding connective tissue. From this, the vagina was prepared, measured and evaluated macroscopically. The vagina was then further processed for histological and biomechanical evaluation, as depicted in Figures 1 D and E.

Gross anatomy:

Vaginal diameters were measured. The vaginal length, defined as the distance between the cervix and the hymen, and the width were measured in the narrowest part of the vagina. Also the uterus was weighed, to confirm oestrogenisation[256].

Histology and immunohistochemistry:

Details are in Appendix S4. Vaginal biopsies and explants were stained with Hematoxylin and Eosin (HE) for surface injury and epithelial thickness (ET; µm; 400x magnification)[261]. Masson's Trichrome and Miller were used to semi-quantify collagen and elastin in the lamina propria (400x magnification)[262]. Periodic acid-Schiff (PAS) was used to visualize epithelial glycogen accumulation (400x magnification)[263]. CD34 endothelial cell marker was used for vascularity quantification and alpha smooth muscle actin (α-SMA[263]) for thickness measurement of lamina propria and muscularis (µm; 25xmagnification). From each specimen up to ten photographs were randomly taken, depending on the specimen size. Slides were evaluated with two observers blinded to the treatment group.

Biomechanical testing:

Vaginal samples (4 from each animal, see Figure 1D; size 10 x 10 mm) were mechanically tested in FIBEr1 using planar biaxial test device (*Messphysik-Zwick/Roel*, Fürstenfeld, Austria; Figure S1 A, B). The sample was submersed during the planar biaxial test in physiological solution at 37°C. A displacement-controlled protocol was applied consisting of equibiaxial loading steps of 5%, 10%, 15%, 20% and 25% nominal strain at a rate of 5% per second. To precondition the samples, the same loading cycles were applied five times, while applying a preload of 0.05 N prior to each loading cycle to prevent the sample from sagging. Corresponding to[264], the stiffness modulus at different strain levels *i* were calculated as the slope of the stress-strain curves at those different strains *i*. The slopes were determined after linearly interpolating the experimental data points in a range between *i*-0.5% and *i*+0.5%, with *i* being a strain of 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10% (details Appendix S5).

Experimental animals:

Twenty-four aged parous female sheep were provided by the Zootechnical Institute of the KU Leuven. Age, parity and weight of the animals were comparable (Table S4; details Appendix S6).

Sample size:

The power calculation was based on a previous sheep study[98], in which multiparous ovariectomized sheep had an ET of 43.7µm (SD 20.2) and had an ET of 81.5µm (SD 27.8) after oestrogens. We used the independent t-test with 80% power, $\alpha = 0.05$ and effect size 1.575. Based on this, eight animals were needed in each group. This makes a total of 24 ewes for the experiment.

Allocating animals to experimental groups:

For allocation of study animals an online randomization tool was utilized (https://www.randomizer.org/). Per each time point, all animals were treated and examined in one day. The order in which they were examined and treated was random.

Statistics:

1 Flanders Institute for Biomechanical Experimentation

Normality testing was done by D` Agostino-Pearson test. One-way ANOVA statistical tests were performed with Sidak post hoc tests if the samples were normally distributed. When not normally distributed, Kruskal-Wallis statistical tests were performed with Dunn's post hoc test. 2-way ANOVA was applied to assess parameters measured over time. Data are presented as mean and SD or median and IQR as appropriate. Significance was reached when the condition $p < 0.05$ was fulfilled. Data were processed using GraphPad Prism version 8.40 for Windows (Graph Pad Prism, La Jolla, CA, USA).

Patient and public involvement:

No patients or public were involved.

Funding:

Fotona LASER (Ljubljana, Slovenia) supported this experiment. The contract is handled by Leuven Research and Development. Fotona did not interfere with the design, analysis, and eventual reporting of this experiment, neither does it own the results. LHH and LK were supported by a grant "Progress Q34", Charles University, Prague, Czechia.

RESULTS:

Histology and immunohistochemistry:

The thickness of the epithelial layer and glycogen containing layer are displayed in Figures 3 A, Figure 4 and Figure S2 E. Following OVX both decreased till 90d where after they stayed at plateau till 193 days. When oestrogen exposed, the vaginal epithelium was significantly thicker (Figure 4 A, D) than in the sham (Figure 4 C, F) and LASER groups (Figure 4 B, E). Animals treated with LASER showed an increase by around one third in the vaginal epithelial thickness (190d) compared to baseline (180d), which was sustained in later vaginal biopsies (257d). The extent by which this was increased was comparable to that measured in sham manipulated animals (Figure 3 A; 67% and 69% at 257 d respectively). In oestrogen-exposed ewes the increase in ET was 202% (257d), which is significantly higher compared to that observed in the two other treatment groups.

The thickness of the glycogen positive layer followed the same trends as epithelial thickness (Figure S2 E). Figure S3 A, B shows values of the lamina propria and lamina muscularis layers, which were no different between the treatment groups. Composition of the lamina propria, in terms of elastin and collagen content, and vascularization did not show any significant differences (Figure S3 C, D, E).

Biomechanical testing:

Vaginal tissue from the oestrogen treated animals was more compliant in both axes ($p < .05$) compared to the other two groups. No difference was observed between the sham and LASER group (Figure 3 B, C).

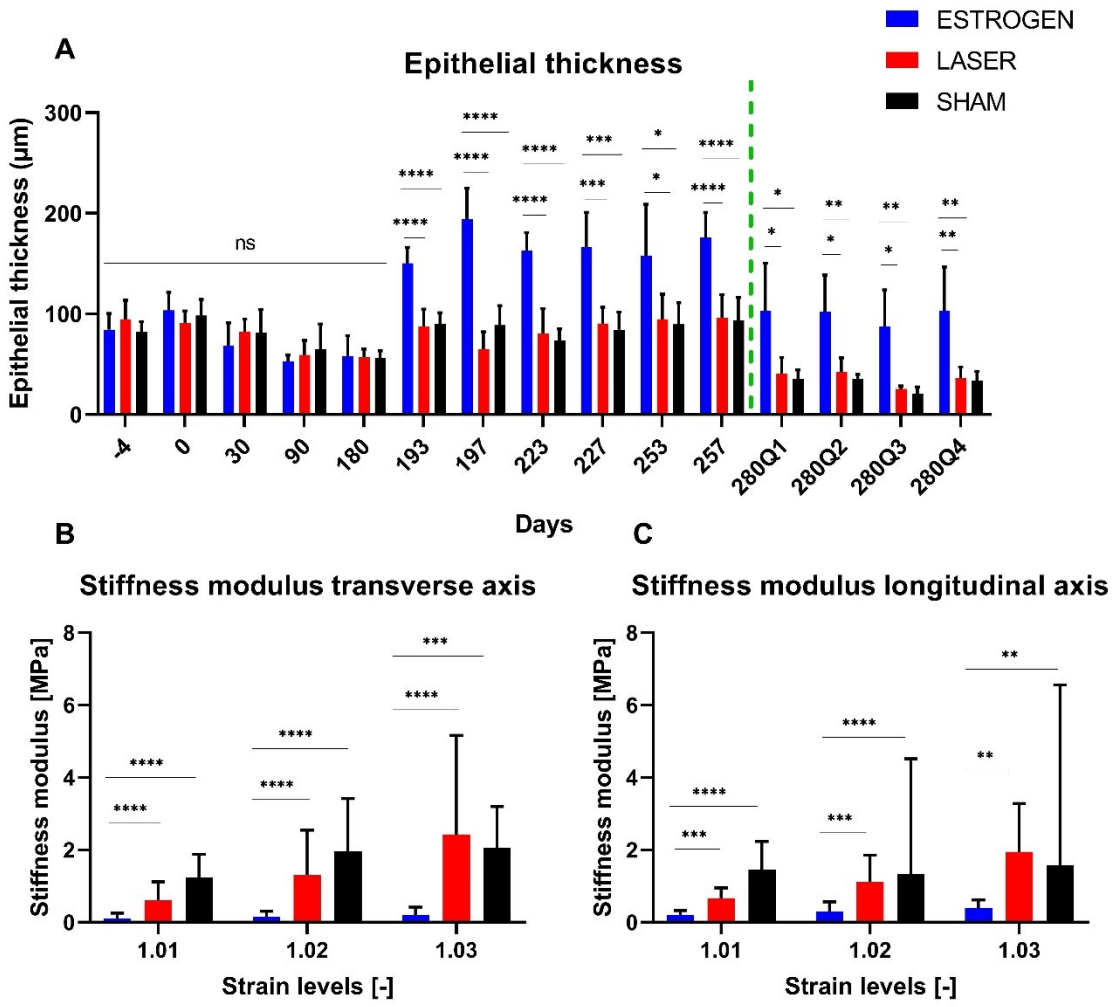

Figure 3. Top row: A. Vaginal epithelial thickness (µm), displayed as mean ± SD. Asterisks mark significance * p < .05 ** p < .01 *** p < .001 **** p < .0001. The green dotted line demarcates the end of the experiment, beyond which necropsy was done. Of note is that the area of the vagina beyond that point, is a different one from the area where the punch biopsies were taken **Bottom row:** Biomechanical properties of the vagina in **B:** the transverse and **C:** longitudinal axis, by means of the stiffness moduli at increasing strains (1% to 3%) displayed as median ± IQR. Asterisks mark significance * p < .05 ** p < .01 *** p < .001 **** p < .0001

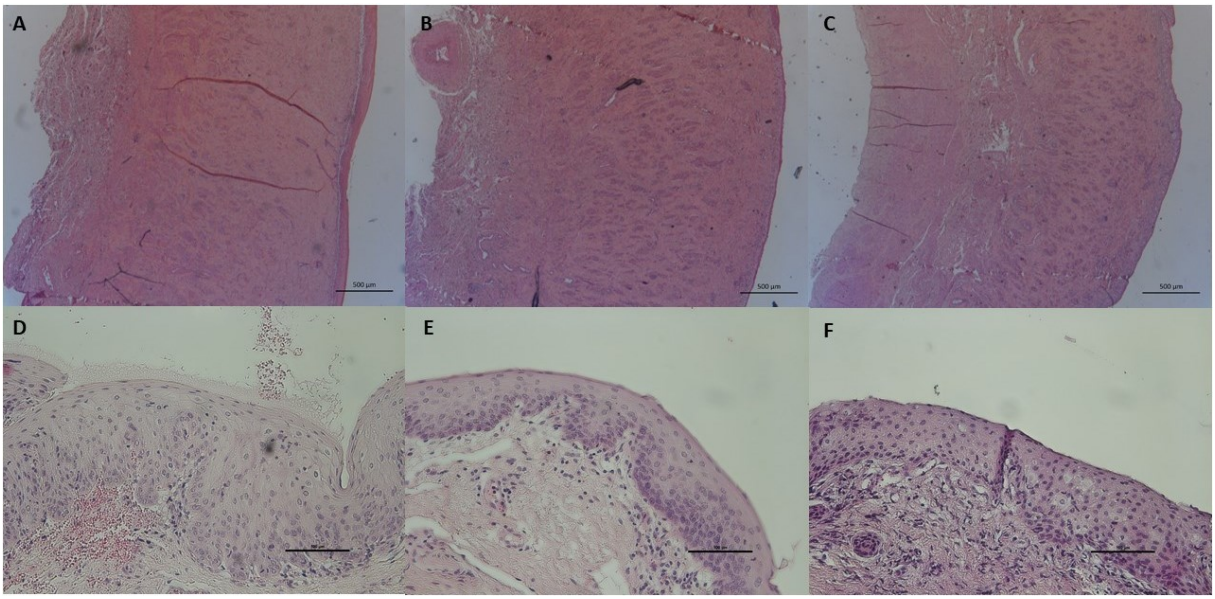

Figure 4. Top row: Representative photographs of full thickness vaginal explants (280d) stained with HE at 25x magnification. From left to right on vaginal sections, one can see the following layers: adventitia, lamina mucsularis, lamina propria and epithelium in **A:** oestrogen, **B:** LASER and **C:** sham group. On the upper left part of photograph B, a vessel in adventitia is present. **Bottom row:** Representative photographs of vaginal epithelium from punch biopsy (257d) stained with HE at 200x magnification. **D:** oestrogen, **E**: LASER and F: sham group.

Clinical examination:

Following OVX, the vagina appeared drier from 90d onwards in all ewes. In the oestrogen group, the vagina appeared more lubricated again from 190d onwards (Figure 2 A), in contrast to sham animals (Figure 2 C). In the LASER group, there was a whitish thick non-offensive discharge in the first 7 days after each procedure (Figure 2 B). The vaginal pH was lower in the oestrogen group compared to the LASER and sham at two time points (193d and 280d) (Figure S2 A). After the second LASER application (d220), there were signs of thermal injury in the vagina (Figure 2 D, E), resolving spontaneously within seven days. As would clinically be done, we lowered the LASER setting, and this did not recur after the third application.

Gross anatomy:

There were no differences in vaginal dimensions, though the uterus weighed more in oestrogen exposed ewes (Figure S2 B, C, D).

One out of 24 ewes died due a respiratory infection on d200 (sham). The detailed outcomes of the entire experiment are displayed in Table S5 and S6.

DISCUSSION:

Main findings

Following ovariectomy, ewes developed vaginal atrophy within 90 days, evidenced by a decrease in thickness of the epithelial and glycogen-positive cell layer and lubrication. These changes were reversed by systemic oestrogens, which also increased vaginal compliance. Both sham and LASER-exposed animals displayed a significant but lesser increase in thickness of the epithelial and glycogen-positive cell layer. LASER induced a transient vaginal discharge. In all but one ewe, there were signs of superficial self-healing thermal injury following the second application. None of the interventions modified the composition and thickness of the lamina propria or the vaginal dimensions.

Strengths and limitation.

Strengths are the design including randomization, blinding of the assessor, appropriate sample size, and positive (oestrogen) as well as a sham control group. Also, the long timeframe (> 9 months) allowed to clearly demonstrate menopausal changes prior to further interventions. Three LASER applications one month apart were done as used clinically. Next to being a well-studied model for menopause, the dimensions of the ovine vagina allowed us to perform repeated biopsies[98, 265]. Further, we are familiar with the ovine model and the measurements used[98, 262, 265]. Biomechanical measurements were expanded to bi-axial testing in a wet environment with repetitive straining, which is more physiologic[266].

The main limitation is that in sheep one cannot measure "subjective" bother of GSM, hence cannot simulate what improvement following LASER treatment women would experience. Though in sheep oestrogen replacement alleviates clinically relevant menopause-induced changes (epithelial thickness, glycogen content), clinical bother is obviously also driven by vaginal dryness, dyspareunia, itching or burning, which are not captured by the read outs used in this experiment. As in previous experiments[36, 39], we chose epithelial thickness as primary outcome measure, but did not observe an additional effect of LASER to the effect of sham manipulation. Another limitation is that we used the LASER settings as in clinic. In pathological studies in menopausal women, epithelial thickness ranges between 50-180 µm[267-269], whereas in sheep it is slightly less (180d; mean 59.1 µm; range: 34.2 - 118.0 µm). This may explain, in part, the thermal impact we observed. To avoid this, we adjusted the energy for the third application (fluence: 3J/cm^2 to 1.75J/cm^2). Apart from the epithelial effects, we did not demonstrate changes in the deeper layers[39, 270]. Also, we tested only Er:YAG LASER, though CO_2-LASER claims a thermal action mechanism as well. In this experiment we also came across the shortcomings of pH-measurements in sheep. Although relevant in women, in sheep vaginal pH is different. Ewes have the urethral opening in the lower third of the vagina (Figure 2 A), hence urine (pH=7.9±0.5[271]) contaminates vaginal secretions. Other clinical factors difficult to assess were vaginal elasticity and epithelial health, and as a consequence, the vaginal health index (containing these variables) does not seem to be a translatable outcome measure, hence we disregarded its results.

Interpretation:

The introduction of novel devices or procedures best follows a sequence of cautious pre-clinical and well controlled clinical trials. The need for that was previously demonstrated by the overzealous introduction of vaginal mesh which led to their eventual withdrawal from the market[272]. LASER treatment remains controversial as there is no experimental basis to prove its effects[260, 273]. Pre-clinical experimentation can provide information about the mechanisms of actions as well as the potential side effects[24, 273]. Herein we measured the effects of Er:YAG LASER application in menopausal sheep. Ovariectomized sheep display changes similar to menopausal women, including osteoporosis[274], hot flushes[275], atherosclerosis[255] and, to our purpose, also vaginal atrophy[98]. We earlier quantified the effects of LASER on the vagina of menopausal sheep, demonstrating a significant increase of epithelial thickness seven days after first LASER application (93±3.6 µm vs 71.4±5.2 µm in sham; p<.05)[46]. However, by 30 days after the third LASER application there were no demonstrable changes in epithelial thickness neither biomechanical properties visible. Because of the potential shortcomings of the design of the previous study, we modified the design in this study to include baseline measurements, a longer hypoestrogenic state, serial biopsies and physical examinations. We also included positive controls, i.e. ewes that were exposed to oestrogen which modified many of the outcome parameters[262].

In this *experimental study*, LASER induced a 69%-increase in epithelial thickness seven days following the third LASER. An earlier clinical study from Gaspar (2017) in women with GSM treated with Er:YAG LASER also reported an "increase in epithelial thickness", persisting up to one year. At closer look however, the authors did not report exact measurements in their methodology. Also, those women applied oestrogens for two weeks *prior* to LASER[36]. In a short report on women not using oestrogens four months prior to LASER, Gaspar[252] observed an

increase in epithelial thickness from 45±33 µm to 153±45µm (+240% from baseline) three months after second LASER application, and an increase in glycogen and neo-angiogenesis. No details on statistics were provided and no control group was used. Another study by Lapii (2017) reported also an increase (64%) in epithelial thickness two months after Er:YAG LASER treatment, though these women had stress incontinence, and not all were menopausal (average: 49 years)[39]. When searching for clinical studies with CO_2 LASER, three[40, 276, 277] reported an increase in ET, however one was describing an "increase" in epithelial thickness on histology in five women, however without providing exact measurements or statistics[276]. The second study did not have an accurate description of the methodology[277], and the last one reported on biopsies taken immediately after LASER[40]

Another remarkable finding was that the increase in ET was within the same range as what was measured in sham manipulated animals (67 %). This observation is interesting, and it is unclear why this occurs. One earlier clinical study showed that vaginal manipulation, either by intercourse or other forms of penetration, two or more times per week, is increasing the "vaginal atrophy index". In that study, no biopsies were taken, so we do not know whether, and how much the ET would have increased[29]. For reference, in our study vaginal manipulations in the sham group included the introduction of the glass speculum (once per month), as well as a clinical speculum for follow-up measurements (twice per month), both lasting around 5 min. This is shorter than vaginal intercourse (7 min[278]) and less frequent (≥2/week)[29]. It is therefore not clear what the exact mechanism is. In an experimental study in menopausal rats, a mechanosensitive pathway was described by analysis of adenosine triphosphate from vaginal washes after vaginal stimulation[279].

Conversely, ewes exposed to systemic oestrogens displayed a *three-fold larger* increase in epithelial thickness (202%). Again, in the above-mentioned clinical study by Gaspar (2017), comparing Er:YAG LASER with topical oestrogens, there is mention of an increased ET, but unfortunately, they did not quantify the magnitude of changes[36].

There are however three clinical RCTs[45-47] that compared another type of LASER (CO_2) to topical oestrogens in GSM patients. None unfortunately included vaginal biopsies, which makes comparison difficult. On the other hand they all reported subjective improvement following laser, in one the improvement in vaginal dryness was comparable[45], in one the VHI increased more[46] and in a third with a similar increase in VHI, laser relieved dyspareunia, burning and dryness, whereas oestrogens only improved dryness[47]. Only one study was double-blind, and none used sham controls. Apart from that criticism, in a functional condition as GSM probably subjective outcomes are very relevant outcome measures. In our experimental study, this was not possible, though we observed a significant increase in glycogen content, lubrication and vaginal compliance, following oestrogens, which did not occur in animals undergoing LASER.

CONCLUSION:

In a well-documented menopause model and with exhaustive methodology we documented similar effects of sham vaginal manipulation and vaginal laser treatment, which were less than in oestrogen substituted animals. These findings await translation in a clinical study of similar design.

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Disclosure of interest:

Fotona LASER (Ljubljana, Slovenia) supported this investigator-initiated experiment through a service agreement. The contract was handled by Leuven Research and Development. Fotona did not interfere with the design, conduct and reporting of this experiment, neither does it own the results.

Author's role:

This study was designed, and its protocol was prepared by K.M., A.M.M., M.G.M.C.M.D.C., L. H.H., I.U., A.W.K., E.V., K.V.L., H.F., Z.G., J.P.R., L.K., J.V., and J.D. Ovariectomy was performed by K.M., M.G.M.C.M.D.C., A.M.M. and A.K. Clinical examinations and biopsies were performed by K.M., A.M.M., I.U., A.K., E.V., Z.G. Histology and immunohistochemistry was performed by K.M. and L.H.H., biomechanical testing was performed by K.M. and K.V.L. Analysis of data and statistical analyses were performed by K.M., L.H.H., K.V.L., H.F., assisted by J.P.R., L.K., J.D. The original manuscript was drafted by K.M. under supervision of J.D. All authors critically reviewed and revised the manuscript and approved the final version.

Details of ethical approval:

This animal experiment was approved on 6th March 2018 by the Animal Ethics Committee of the KU Leuven (registration number: P038/2018) in accordance with the Animals (Scientific Procedures) Act 1986 and performed in compliance with the EU-regulations as declared in Directive 2010/63/EU and the Belgian Royal Decree of 29 May 2013.

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SUPPLEMENT:

Supplementary Figures:

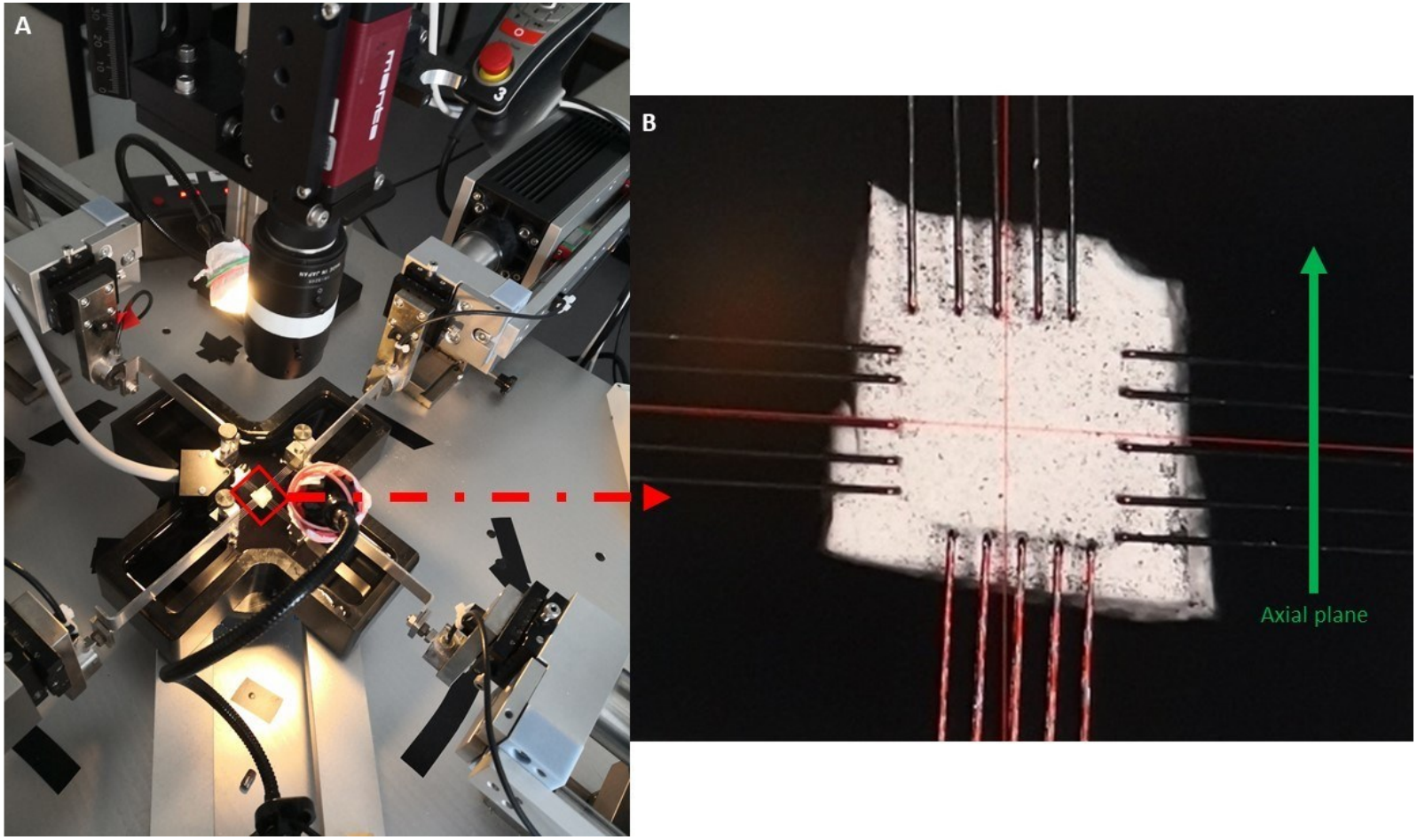

Figure S1. A: Sample mounted on the rakes and submersed into physiological solution. **B:** Detail of the sample with graphite pattern.

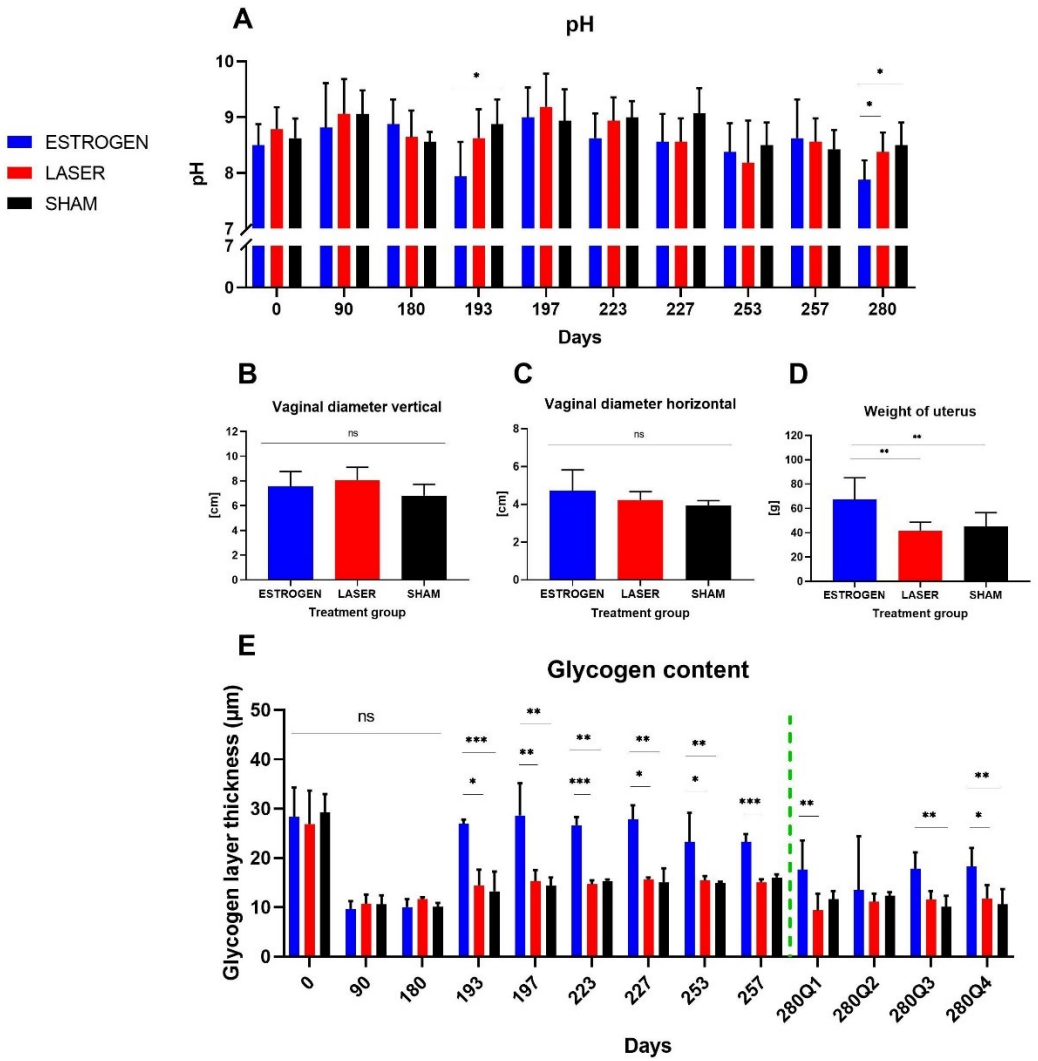

Figure S2. Top row: A. Vaginal pH measurements throughout the experiment (mean ± SD. Asterisks marks significance * $p < .05$). **Middle row:** Gross anatomical findings in the vagina. **B:** is the vertical and **C:** the horizontal diameter, and **D:** is the uterine weight. Measurements are displayed as mean ± SD. Asterisks mark significance ** $p < .01$. **Bottom row: E.** Thickness of layer with glycogen positive cells (µm). Bars represent median ± IQR. Asterisks marks significance * $p < .05$ ** $p < .01$ *** $p < .001$

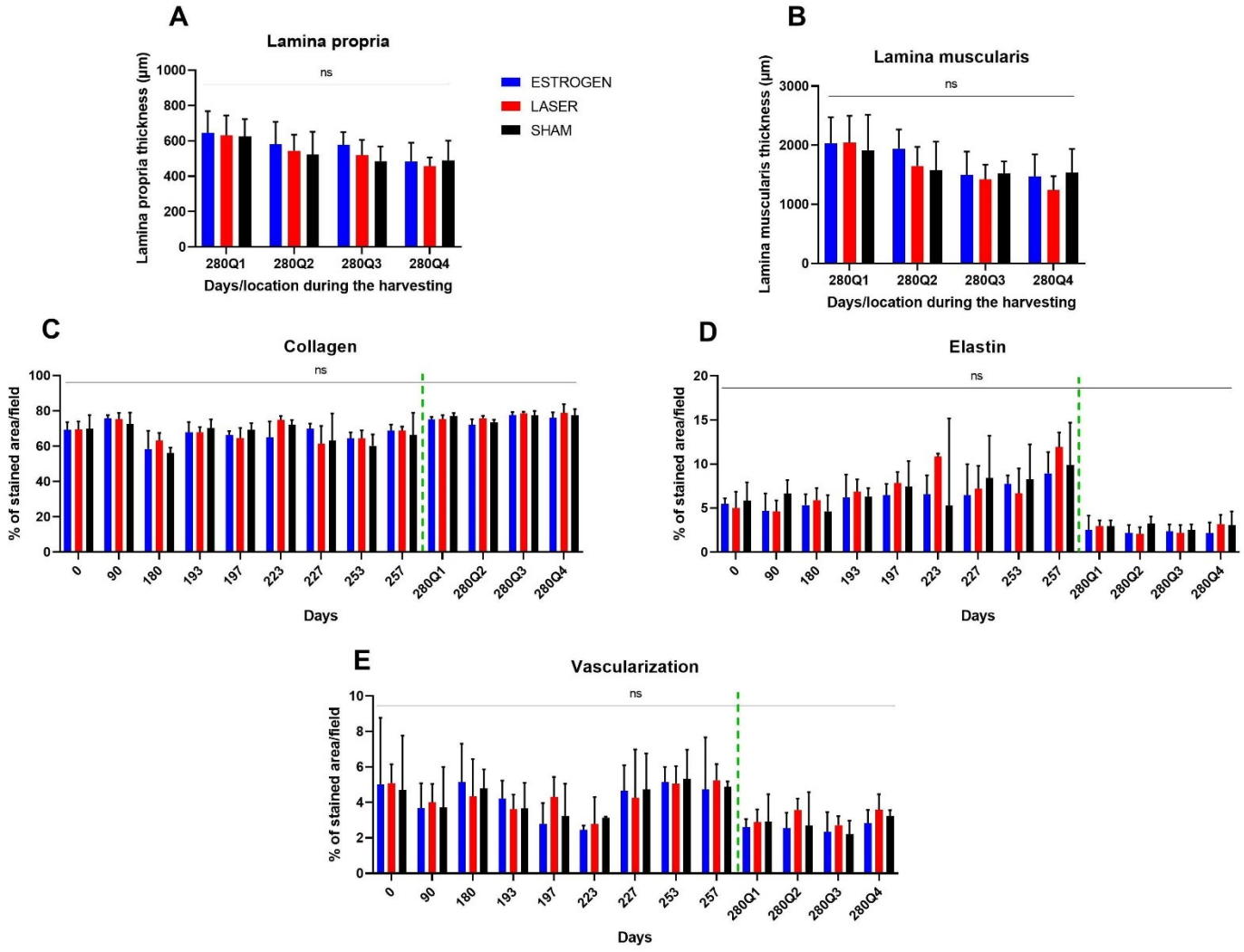

Figure S3. Top row: Graphs displaying thickness of the **A:** lamina propria and **B:** lamina muscularis (µm, both displayed as mean ± SD) **Middle row:** Graphs displaying the density of **C:** collagen and **D:** elastin; (% of stained area/field, both displayed as median ± IQR) **Bottom row: E.** Graph displaying endothelial cells (% of stained area/field; median ± IQR). ns = not significant.

Supplemetary tables:

Table S1. Time course of the experiments and interventions.

Days to ovariectomy	-18	-4	0	30	90	180	190	193	197	220	223	227	250	253	257	280
Synchronization /sponge	in	out														
Bilateral ovariectomy			X													
Laser							X			X			X			
Estrogen implantation							X									
Punch biopsy/necropsy		X	X	X	X	X		X	X		X	X		X	X	X
pH			X		X	X		X	X		X	X		X	X	X
Vaginal health index			X		X	X		X	X		X	X		X	X	X
Euthanasia and terminal measurements																X

Table S2. Laser protocol settings.

SMOOTH protocol	Wavelength (nm)	spot size (mm)	Fluence (J/cm²)	Pulses (n)	ejection steps distance (mm)	Passes (n)
IntimaLase™	2940	7	3	7	5	5
RenovaLase™	2940	7	1.75	7	5	5

Table S3. Vaginal health index (VHI)[280]

	Score	1	2	3	4	5
	Elasticity	None	Poor	Fair	good	excellent
Volume	Fluid	None	scant amount, vault not entirely covered	scant amount, vault entirely covered	moderate amount	normal amount
	pH*	9 and more	8.5	8	7.5	7 and less
injury	Epithelial	petechiae noted before contact	bleeds with light contact	bleeds with scraping	not friable-thin epithelium	normal
	Moisture	none, surface inflamed	none, surface not inflamed	minimal	moderate	normal

*The clinical pH scale was adjusted to the findings in menopausal sheep. Reproductive ewes have, on average, a vaginal pH of 6.7±0.4[281]. Postmenopausal sheep had a higher pH, ranging from 7.875 up to 9.188.

Table S4. Characteristics of the ewes at the onset of the experiments. Age is given as median and IQR, weight and parity are given as mean and SD.

Treatment group	Number	Age (years) (median ± IQR)	Weight (kg) (mean ± SD)	Parity (n) (mean ± SD)
Er: YAG Laser	8	8 ± 0.0	63.38 ± 9.58	1.875 ± 0.64
Estrogen	8	8 ± 0.75	60.13± 10.40	1.625 ± 0.52
Sham	8	8.5 ± 1.75	65.71 ± 6.40	1.625 ± 0.52

Table S5. Results from punch biopsies and from specimens at harvesting. Normally distributed data are displayed as mean ± standard deviation, the others as median ± interquartile range. Those rows are indicated by the "#" sign.

Days to OVX	**-4**			**0**			**30**			**90**			**180**		
Group	**Estrogen**	**Laser**	**Sham**	**Estrogen**	**Laser**	**Sham**	**Estrogen**	**Laser**	**Sham**	**Estrogen**	**Laser**	**Sham**	**Estrogen**	**Laser**	**Sham**
Epithelial thickness (µm)	84.41± 15.84	94.54± 19.17	81.90± 10.38	103.70± 17.65	91.21± 11.76	98.45± 15.99	68.54± 22.56	82.27± 12.72	81.33± 23.05	52.94± 6.10	59.23± 14.29	65.13± 24.80	58.04± 20.27	56.78± 8.29	56.14± 7.21
Glycogen thickness (µm) #	-	-	-	28.31± 16.95	26.90± 12.99	29.30± 10.25	-	-	-	9.71± 1.97	10.71± 3.07	10.62± 3.59	10.07± 2.90	11.63± 1.55	10.10± 1.39
Collagen (%area) #	-	-	-	69.25± 10.45	69.40± 7.39	69.88± 15.85	-	-	-	75.74± 3.40	75.23± 11.33	72.46± 9.40	58.38± 17.52	63.14± 8.70	56.24± 4.73
Elastin (%area) #	-	-	-	5.48± 1.28	5.00± 2.66	5.85± 4.43	-	-	-	4.69± 3.24	4.61± 2.80	6.63± 3.17	5.34± 1.73	5.91± 2.45	4.65± 2.89
Vascularization (%area) #	-	-	-	5.02± 4.55	5.09± 1.78	4.70± 4.34	-	-	-	3.69± 2.92	4.01± 2.77	3.74± 3.91	5.15± 2.80	4.34± 2.80	4.80± 2.82
pH	-	-	-	8.50± 0.38	8.79± 0.39	8.63± 0.35	-	-	-	8.81± 0.80	9.06± 0.62	9.06± 0.42	8.88± 0.44	8.64± 0.48	8.56± 0.18
Days to OVX	**193**			**197**			**223**			**227**			**253**		
Group	**Estrogen**	**Laser**	**Sham**	**Estrogen**	**Laser**	**Sham**	**Estrogen**	**Laser**	**Sham**	**Estrogen**	**Laser**	**Sham**	**Estrogen**	**Laser**	**Sham**
Epithelial thickness (µm)	150.40± 15.72	87.44± 17.20	90.31± 10.70	194.20± 30.60	64.81± 17.21	88.89± 19.34	162.80± 17.97	80.53± 24.74	73.57± 11.49	166.10± 34.69	90.35± 16.27	84.42± 17.55	158.00± 50.90	94.63± 24.94	89.52± 21.55
Glycogen thickness (µm) #	26.99± 1.93	14.43± 4.48	13.29± 6.08	28.64± 9.31	15.30± 4.51	14.34± 2.84	26.65± 3.71	14.68± 1.36	15.37± 1.33	27.81± 7.08	15.65± 2.11	15.09± 5.65	23.27± 8.95	15.50± 2.69	15.04± 1.38
Collagen (%area) #	67.76± 12.86	67.83± 8.12	70.13± 11.57	66.26± 4.76	64.45± 10.68	69.23± 7.75	64.96± 10.37	74.82± 10.76	72.07± 4.71	69.86± 14.15	61.31± 12.43	63.24± 19.87	64.31± 8.87	64.34± 7.81	60.18± 10.62
Elastin (%area) #	6.21± 3.80	6.85± 2.52	6.32± 3.05	6.45± 2.28	7.84± 3.53	7.44± 3.63	6.58± 4.02	10.83± 3.61	5.29± 10.46	6.46± 5.66	7.20± 3.50	8.41± 9.11	7.75± 2.62	6.67± 3.83	8.26± 8.43
Vascularization (%area) #	4.23± 2.23	3.62± 2.33	3.66± 2.39	2.79± 1.92	4.32± 2.06	3.24± 2.89	2.45± 0.54	2.78± 2.44	3.14± 1.05	4.66± 1.65	4.27± 3.37	4.72± 2.55	5.15± 1.26	5.05± 2.54	5.33± 3.53
pH	7.94± 0.62	8.63± 0.52	8.88± 0.44	9.00± 0.53	9.19± 0.59	8.94± 0.56	8.63± 0.44	8.94± 0.42	9.00± 0.29	8.56± 0.50	8.56± 0.42	9.07± 0.45	8.38± 0.52	8.19± 0.75	8.50± 0.41
Days to OVX	**257**			**280 Q1**			**280 Q2**			**280 Q3**			**280 Q4**		
Group	**Estrogen**	**Laser**	**Sham**	**Estrogen**	**Laser**	**Sham**	**Estrogen**	**Laser**	**Sham**	**Estrogen**	**Laser**	**Sham**	**Estrogen**	**Laser**	**Sham**
Epithelial thickness (µm)	175.60± 25.28	96.16± 23.03	93.69± 22.74	103.10± 47.36	40.73± 16.00	35.40± 9.04	102.10± 36.45	42.17± 14.29	35.07± 4.84	87.59± 36.22	25.45± 3.02	20.65± 6.77	103.10± 43.43	36.20± 10.92	33.85± 9.10
Glycogen thickness (µm) #	23.31± 4.10	15.09± 2.55	16.06± 1.30	17.70± 10.13	9.38± 4.74	11.70± 1.98	13.48± 12.57	11.13± 2.78	12.38± 3.73	17.83± 8.12	11.60± 3.52	10.10± 4.32	18.27± 6.98	11.77± 4.72	10.62± 4.88
Collagen (%area) #	68.72± 7.16	68.71± 6.55	66.49± 16.85	75.06± 4.65	75.22± 5.75	76.84± 8.31	71.93± 3.80	75.81± 3.52	73.45± 4.49	77.63± 2.76	78.66± 4.32	77.46± 6.16	76.02± 5.48	78.78± 8.88	77.30± 7.64
Elastin (%area) #	8.94± 4.51	11.90± 5.41	9.89± 6.35	2.54± 2.78	2.96± 1.78	2.95± 1.27	2.19± 1.69	2.08± 0.94	3.28± 1.90	2.38± 1.92	2.18± 1.79	2.53± 1.42	2.15± 1.44	3.18± 1.18	3.08± 1.94
Vascularization (%area) #	4.72± 3.23	5.25± 2.26	4.89± 1.60	2.62± 1.21	2.89± 0.96	2.92± 2.19	2.55± 1.26	3.58± 1.59	2.69± 2.29	2.34± 1.48	2.72± 0.91	2.22± 1.12	2.84± 1.77	3.59± 1.65	3.24± 0.81
pH	8.63± 0.69	8.56± 0.42	8.43± 0.35	-	-	-	-	-	-	-	-	-	-	-	-
Thickness of lamina propria (µm)	-	-	-	645.80± 123.10	631.70± 112.90	625.60± 98.50	581.40± 126.70	544.60± 89.58	521.80± 130.30	577.90± 72.23	519.40± 86.90	486.30± 82.25	483.40± 106.50	457.00± 50.13	490.90± 111.50
Thickness of lamina muscularis (µm)	-	-	-	2025.00± 449.30	2047.00± 448.90	1914.00± 603.50	1941.00± 325.10	1649.00± 322.10	1576.00± 482.30	1498.00± 395.90	1422.00± 249.80	1522.00± 202.60	1471.00± 374.80	1238.00± 237.70	1536.00± 399.80

Table S6. Results from specimens at harvesting. Normally distributed data are displayed as mean ± standard deviation, the others as median ± interquartile range. Those rows are indicated by the "#" sign.

Days to OVX					**280 (harvesting)**				
Group		**Estrogen**			**Laser**			**Sham**	
pH		7.88± 0.35			8.38± 0.35			8.50± 0.41	
Vaginal diameters vertical (cm)		7.58± 1.19			8.06± 1.06			6.81± 0.91	
Vaginal diameters horizontal (cm)		4.73± 1.10			4.23± 0.45			3.94± 0.25	
Weight of uterus (g)		67.25± 17.90			41.63± 7.07			45.14± 11.39	
	Strain 1.01	*Strain 1.02*	*Strain 1.03*	*Strain 1.01*	*Strain 1.02*	*Strain 1.03*	*Strain 1.01*	*Strain 1.02*	*Strain 1.03*
Stiffness modulus transverse axis (MPa) #	0.11± 0.18	0.15± 0.22	0.21± 0.32	0.61± 0.68	1.30± 1.72	2.43± 3.70	1.24± 1.43	1.96± 2.68	2.10± 2.40
Stiffness modulus longitudinal axis (MPa) #	0.21± 0.21	0.29± 0.41	0.40± 0.42	0.66± 0.46	1.12± 1.31	1.94± 2.50	1.45± 1.80	1.32± 3.86	1.58± 5.78

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Appendix S1. Hormonal replacement therapy

The release system was prepared from silastic tubing (0.335 cm inside and 0.465 cm outside diameter; Dow Corning, Midland MI) and filled with pure crystalline 17β oestradiol, sealed on both ends with silicon glue (732 multipurpose sealant; Dow Corning, Midland MI). The capsule's length was 5 cm (with 0.5 cm glue plug on each side). The capsule, when placed under the skin, has been shown to maintain a physiological serum E2 concentration and produce a serum E2 level of 5 pg/mL with a rate of release of approximately 1.5 pg/day[275, 282, 283]. Prior to insertion, the implants were equilibrated in 0.1% albumin buffer (2% FBS in PBS) for 24 h in 37°C to prevent a transient postinsertion peak in circulating steroid[284].

Appendix S2. Bilateral ovariectomy

After sedation with a intramuscular injection of xylazine and tramadol (0.03 mg/kg Xyl -M®; VMD; Arendonk; Belgium; 0.15 mg/kg Tramadol EG; Eurogenerics, Brussels, Belgium), epidural anesthesia was induced by an mixture of xylocaine with morphine (0.75 mg/kg Xylocaine-1% Adrenaline, AstraZeneca NV, Dilbeek, Belgium; 0.08mg/kg Morphine HCL, Sterop, Brussels, Belgium). Under sterile conditions, from a left flank incision, the uterine horns, tubes and ovaries were identified. The vessels supplying the ovary were ligated with 0 polyglacitne 910 (Vicryl®, Ethicon, Zaventem, Belgium) and ovaries were removed. The peritoneum and abdominal fascia were subsequently closed with 1/0 polydioxanone suture (PDS II 1, Ethicon), the subcutaneous layer and skin were closed with 3/0 poliglecaprone (Monocryl, Ethicon). At the end, the suture was covered with Aluminium spray. After surgery, animals were returned to their stable, allowed to move, eat and drink *ad libitum*. Meloxicam (Metacam®, Boehringer Ingelheim, Rhein, Germany) was applied as an analgesic for three days postoperatively.

Appendix S3. Obduction

Sheep were euthanized by intravenous administration of a mixture of embutramide, mebezonium and tetracaine hydrochloride (T61; MSD, Animal health BVBA; Brussels; Belgium; 4-6 mL / 50 kg) after sedation with xylazine (10 mg/kg) (Xyl -M®; VMD; Arendonk; Belgium), administered intramuscularly[285].

Appendix S4. Histology and immunohistochemistry

Vaginal biopsies and explants were fixed in 4% paraformaldehyde for 24h, then immersed in phosphate buffered saline (PBS) for one hour, stored in 70% ethanol, embedded in paraffin, cut in five µm sections and stained with Hematoxylin & Eosin (H&E), Masson's Trichrome, Miller, Periodic acid-Schiff (PAS), CD34 and alpha smooth muscle actin (α-SMA). Images were captured with Carl Zeiss Axioskop (Oberkochen; Germany) at 25x and with Nicon Eclipse Ci (Melville, NY; U.S.A.) at 400x magnification. From each specimen up to ten photographs were randomly taken, depending on the specimen size. Slides were evaluated with two observers blinded to the treatment group. For thickness measurements, two software were used: ZEN II lite (Carl Zeiss; Oberkochen; Germany) and NIS-Elements 4.60 (Nicon; Melville, NY; U.S.A.). For the morphometric analysis, Fiji software[286] was used. H&E staining was used was for surface injury and epithelial thickness (ET; µm; 400x magnification)[261]. Masson's Trichrome and Miller were used to semi-quantify collagen and elastin in the lamina propria (400x magnification)[262]. PAS was used to visualize epithelial glycogen accumulation (400x magnification)[263]. Vascularity was quantified by endothelial cell marker CD34. Thickness (µm; 400x magnification) of lamina propria and lamina muscularis were measured from the full thickness specimen (25x magnification) in slides stained with α-SMA[263].

Appendix S5. Biomechanical testing

Vaginal samples (4 from each animal, see Figure 1D; size 10 x 10 mm) were mechanically tested in FIBEr2. The samples were stored in a physiological solution at -80°C. Prior to mechanical testing, the tissue was thawed overnight at 4°C. Sample thickness was measured optically by means of a calibrated picture of the side of the sample using a 12.2 MP *Canon* camera. Graphite powder was used to apply a speckle pattern on the epithelial side of the explant to conduct digital image correlation (DIC). Rakes (5 needles with a diameter of 0.3 mm and a bended tip of 2 mm, spaced 1 mm apart) were used to mount the sample on a planar biaxial test device (*Messphysik-Zwick/Roel*, Fürstenfeld, Austria; Figure S1 A, B). The sample was submersed during the planar biaxial test in physiological solution at 37°C and manually mounted on the rakes, while aligning the principal experimental axes with the longitudinal and transverse sample axis. The initial rake to rake separation at the starting position was 6 mm. A displacement-controlled protocol was applied consisting of equibiaxial loading steps of 5%, 10%, 15%, 20% and 25% nominal strain at a rate of 5% per second. To precondition the samples, the same loading cycles were applied five times, while applying a preload of 0.05 N prior to each loading cycle to prevent the sample from sagging. The fifth loading cycle of the last completed loading step was used for further processing. Force data was stored at 20 Hz. The first Piola-Kirchhof stress was calculated by dividing the average force measured by two opposing actuators by the initial cross-sectional area, the latter being the product of the thickness and the rake-to-rake distance when reaching the preload. One 9.2 MP *Manta* G-917-B camera (*Allied Vision Technologies*, Stadtroda, Germany) with a *Sony* ICX814 CCD image sensor (Tokyo, Japan) was positioned perpendicularly to the sample's surface. A *Kowa* lens (Soka-shi, Japan) with a focal length of 50 mm and 20 mm extension tube was used to visualize the sample. The camera's sample rate was 20 Hz. Deformation was tracked by performing DIC (Vic-2D, *Correlated Solutions*, South Carolina, USA) on the central 25% of the area in between the rakes, after which the nominal strain in both experimental directions was calculated. Corresponding to[264], the stiffness modulus at different strain levels *i* were calculated as the slope of the stress-strain curves at those different strains *i*. The slopes were determined after linearly interpolating the experimental data points in a range between *i*-0.5% and *i*+0.5%, with *i* being a strain of 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10%.

Appendix S6. Housing and husbandry

Ewes were subject to veterinary inspection prior to use. They were housed in group pens on deep straw bedding in a naturally ventilated and lit barn and fed a maintenance diet of *ad libitum*. Feed which may contain phyto-oestrogens (soya, red clove or similar) was avoided during the experiment, to avoid interference with the experimental treatment[287].

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Chapter 7

Does the polymer matter in textile implants for reconstructive surgery?

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ABSTRACT

Implants are crucial in reconstructive surgery, to decrease recurrence rate and enable reconstructions of defects that would otherwise be inoperable. However, their use potentially induces adverse events (AE). Patient and surgical factors, though also material properties, including weight, pore size, textile structure and way of manufacturing, may play a causative role. Animal experimental studies are frequently used to better understand the pathogenesis of AE, though often several variables are tested simultaneously. We designed an experiment in a rat incisional hernia model, wherein two implants with identical textile structure were compared, and that only differed by the polymer they were made off. Tested polymers were polypropylene (PP), which is most widely used, and polyvinylidene fluoride (PVDF), proposed for its milder host response and higher compliance. Passive biomechanical testing of explants revealed no differences between the two polymers. Only at one time-point (90d), explant with PP was stiffer compared to SHAM operated animals. The rate of clinical complications was very low for both implants. Differences in gene expressions, histology and immunohistochemistry were small. In rats, implants with an identical textile structure, though based on a different polymer with a different weight, induce a comparable host response and tissue integration, without measurable difference in passive biomechanical properties.

INTRODUCTION:

Textile implants, usually referred to as meshes, have been introduced into the field of reconstructive surgery in the second half of the 20th century[47] to reduce the high recurrence rates following "native tissue" repair of defects, i.e. using the patient's own tissue and sutures[48, 49]. First used in abdominal and inguinal hernia surgery, mesh was later applied in the treatment of urinary incontinence and pelvic organ prolapse (POP). The use of mesh decreases the 10-years' recurrence rate after incisional hernia surgery from 63% to 32%[288]. Also, it became possible to operate patients with large, previously inoperable defects[289, 290]. In addition, mesh reduces the recurrence rate following inguinal hernia repair from 17% to 1%[291].

Unfortunately, mesh may induce local short-term and long-term adverse events (AE), including pain or feeling of a foreign body, exposure (often referred to as erosion), extrusion, or infection[51]. Depending on the location of implantation, this may cause functional problems like subfertility, dyspareunia, infection, bladder emptying problems, functional bladder and bowel symptoms.

Eventually, the use of mesh is dependent on the balance of the reduction in risk for recurrence, and the added risk for implant-related complications. For inguinal hernia mesh the risk of AE is 3.6% in the first 3 years, dropping to 0.5% in the next 3 years[292]. Conversely, for vaginally inserted mesh for prolapse repair, the risk for complications is 10%[22, 23, 52]. This is far more than when implants for prolapse are inserted abdominally, e.g. for sacrocolpopexy or rectopexy (0.8%)[53].

Nevertheless, considering the high prevalence of POP, UI, incisional and inguinal hernia, and the need for their surgical correction[55-57, 293, 294], there will be always a need for mesh in reconstructive surgery. Its use may grow as the population ages, live longer and more active lives[58], and unfortunately also includes a higher number of obese patients, who are at increased risks for the conditions above[59, 60]. In view of that, health authorities recommended more research to understand the pathogenesis of mesh complications[61]. Several factors associated with AE have been identified, including patient` characteristics, surgical skills, as well as implant properties, such as mechanical properties, mesh weight, pore size and stability, polymer used as well as the manufacturing process[51].

Today, there seems to be a consensus that implants should be macroporous, light weight, stable, and once incorporated into the host, have mechanical properties close to those of native tissue[47, 62]. These factors are close inter-related but so far, there has been no study that tested the impact of the choice of polymer itself in meshes of otherwise identical structure.

Our aim was hereby to fill this knowledge gap and compare the biomechanical properties, host response and integration of two implants with exactly identical textile structure though made from different polymers, using a rat incisional hernia model[295]. One implant was made from polypropylene (PP), which is most widely used material, whereas the other was polyvinylidene fluoride (PVDF), which has been proposed for its milder host response and higher compliance[296].

As a primary outcome we chose compliance after integration into the host, because it is considered to play a crucial role in the development of local AE. Stiffness and mesh contraction by bridging fibrosis has been named as a cause of pain and loss of function[51]. This may also induce atrophy of underlying muscle[198, 297]. We also measured integration into the host tissue, quantified the host response and local wound complications.

RESULTS:

There was no difference in local complications

In total 21 animals had to be euthanized in first days after surgery because of auto-mutilation (biting out the skin covering the implant and/or the implant itself) (10 in PP; 8 in PVDF; 3 in sham). All were replaced to obtain the anticipated sample size; we also prolonged the duration they carried their Elizabethan collar from 24 to 48h, following which this problem disappeared.

At harvesting, the following local complications were noted (Fig. 1): mesh exposure (PP: 7.1% [3/42]; PVDF 7.4% [3/42]), seroma (PP: 2.4% [1/42]; PVDF: 0%) and folding (PVDF: 2.4% [1/42]). None of the animals showed reherniation.

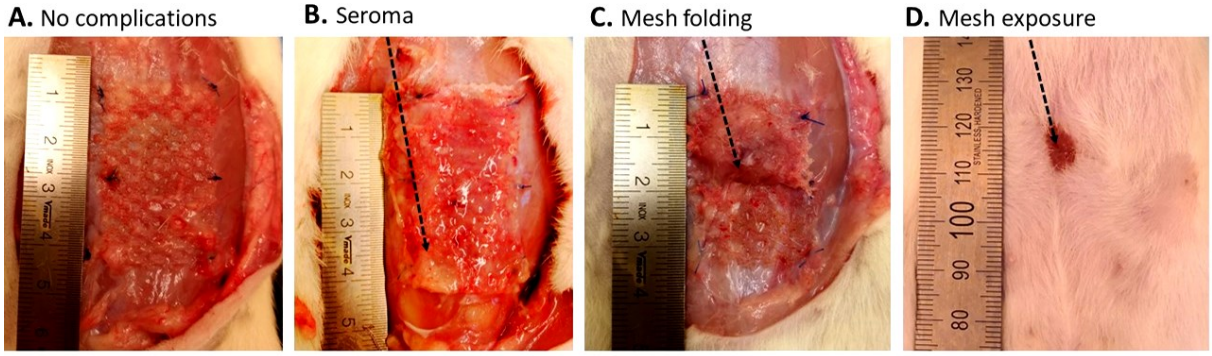

Fig. 1. Representative photograph of obduction. (**A**) Animal with no complication, (**B**) seroma, (**C**) mesh folding, (**D**) mesh exposure. Arrow indicates the pathology.

There was no difference in biomechanical properties between the explants

Even though the preimplantation biomechanical properties differed slightly – PVDF being 6,5% stiffer (p=0,0153; Fig. 2A)-, there was no difference in biomechanical properties of explants (Fig. 5B) when comparing the two materials. 90 days-PP explants were stiffer compared to SHAM operated animals and not operated animals, but not at 180 days or any of the other time points (Fig. 2B).

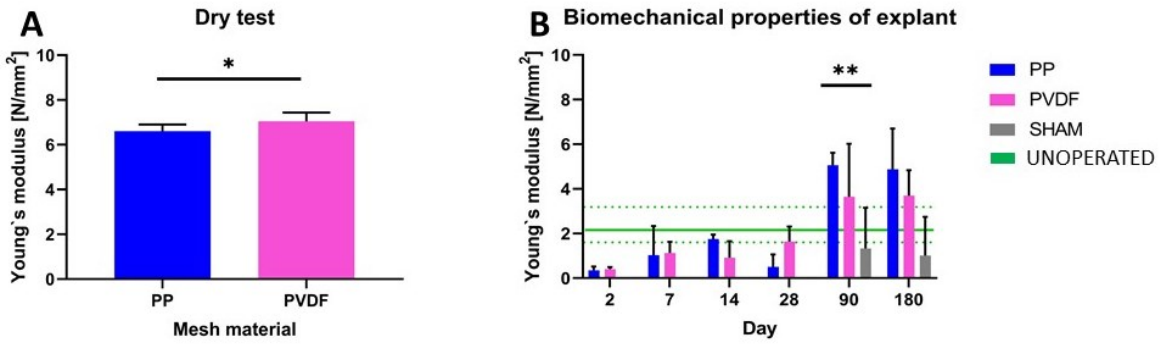

Fig. 2. Graphs displaying results of biomechanical tests. Properties of mesh implants (**A**) before implantation and (**B**) explants from PP, PVDF and sham operated group. Data on graph a are normally distributed, displayed as mean ± SD, unpaired t-test (two-tailed, CI 95%). Data on graph b are not normally distributed, displayed as median ± IQR, Kruskall-wallis test. Green lines show biomechanical properties of abdominal wall from not operated animals (median ± IQR). Asterisks mark significance * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$, ns stands for not significant.

There were small differences between implants in morphometry

Results of morphometry are displayed in Fig. 3.

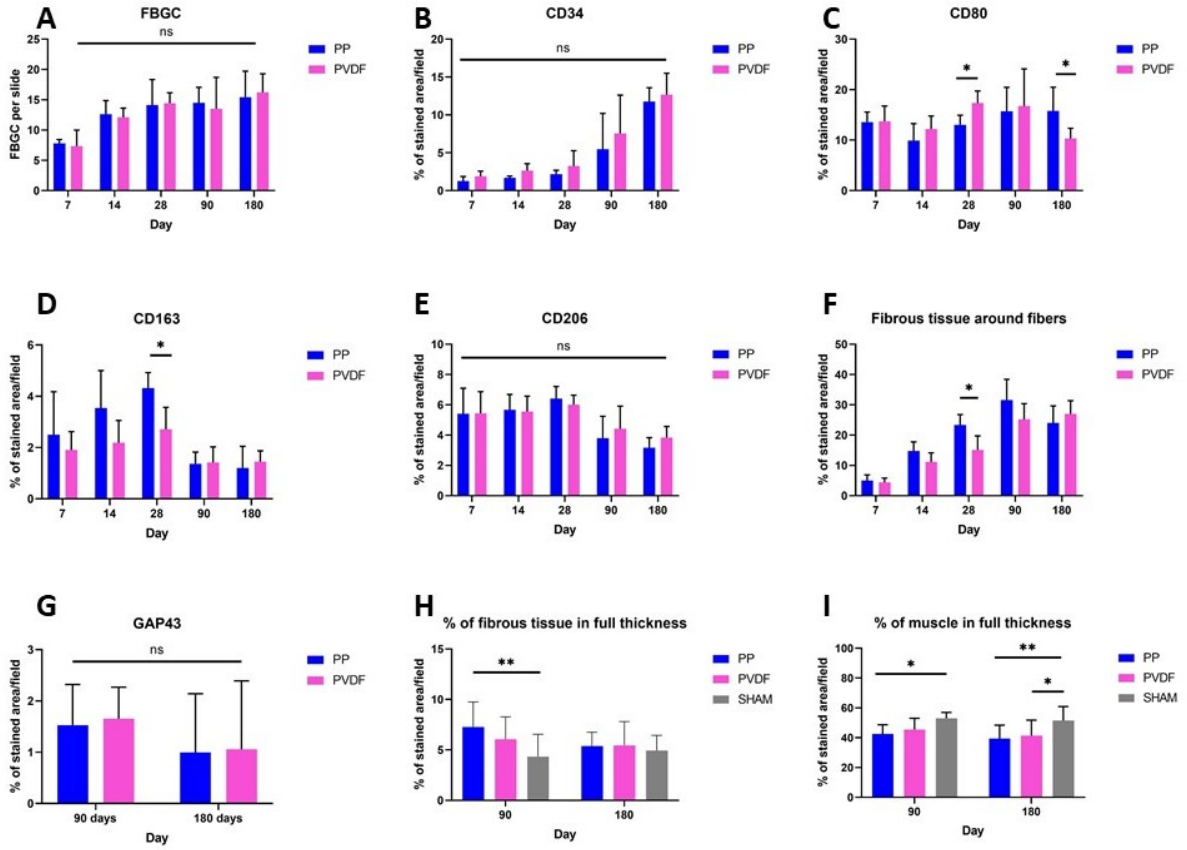

Fig. 3. Graphs displaying histology and immunohistochemistry. **Top row:** (**A**) FBGC, (**B**) CD34, (**C**) CD80, **Middle row:** (**D**) CD163, (**E**) CD206, (**F**) Fibrous tissue around fibers, **Bottom row:** (**G**) GAP43, (**H**) % of fibrous tissue, **(I)** % of muscle. Data are normally distributed, N=5 at day 7, 14, 28 and N=11 at day 90 and 180. Data are displayed as mean ± SD, Mixed effects analysis. Asterisks mark significance * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$, ns stands for not significant.

In the majority of early (day 2) explants, there was no visible implant material, most probably because of lack of integration and subsequent displacement during processing. Therefore, explants harvested early on are not reported.

There were very little differences in inflammation and tissue integration between the two implants. There were some exceptions, such as M1/M2 cell counts at the 28d and 180 d time points, or deposition of fibrous tissue, which was a bit more generous around PP fibers at 28 d. In other words, there were no differences between the two materials in the percentage of muscle (red on Masson Trichrome) and fibrous (blue) tissue at larger magnification. However, when comparing explants at later (90-180 d) time points to SHAM-operated animals, there was more fibrous tissue around the PP implant (90 d), and the muscle thickness in contact with the implant was thinner for both implants.

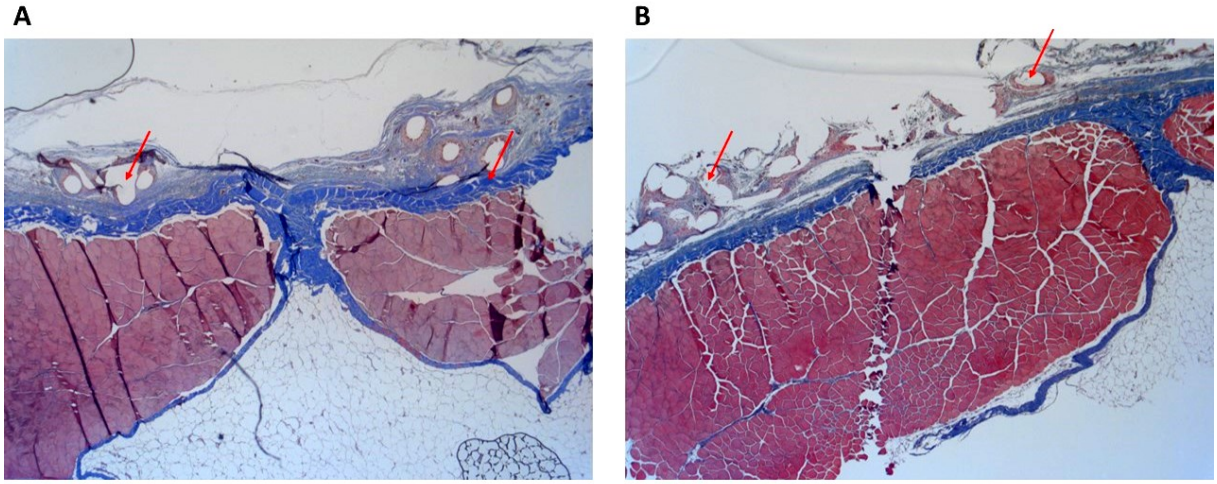

Fig. 4. Full thickness section in 25x magnification stained with Masson trichrome. Representative photos showing ingrowth of host tissue to mesh implant at 90d in (**A**) PP, (**B**) PVDF group. Mesh in depicted by red arrows. From up to down, one can see mesh with connective tissue (blue), fascia (blue) muscle (red), peritoneum (blue).

Differences in gene expression were minimal

Gene expression of 9 selected genes characterizing the host response (Fig. 5), there was only one difference, i.e. TGF-β1 expression was higher in PVDF explants on d7 ($p = 0.0054$). Of note is that this was not paralleled by increased immunoreactivity n TGF-β1 measurement ($p= 0.5292$).

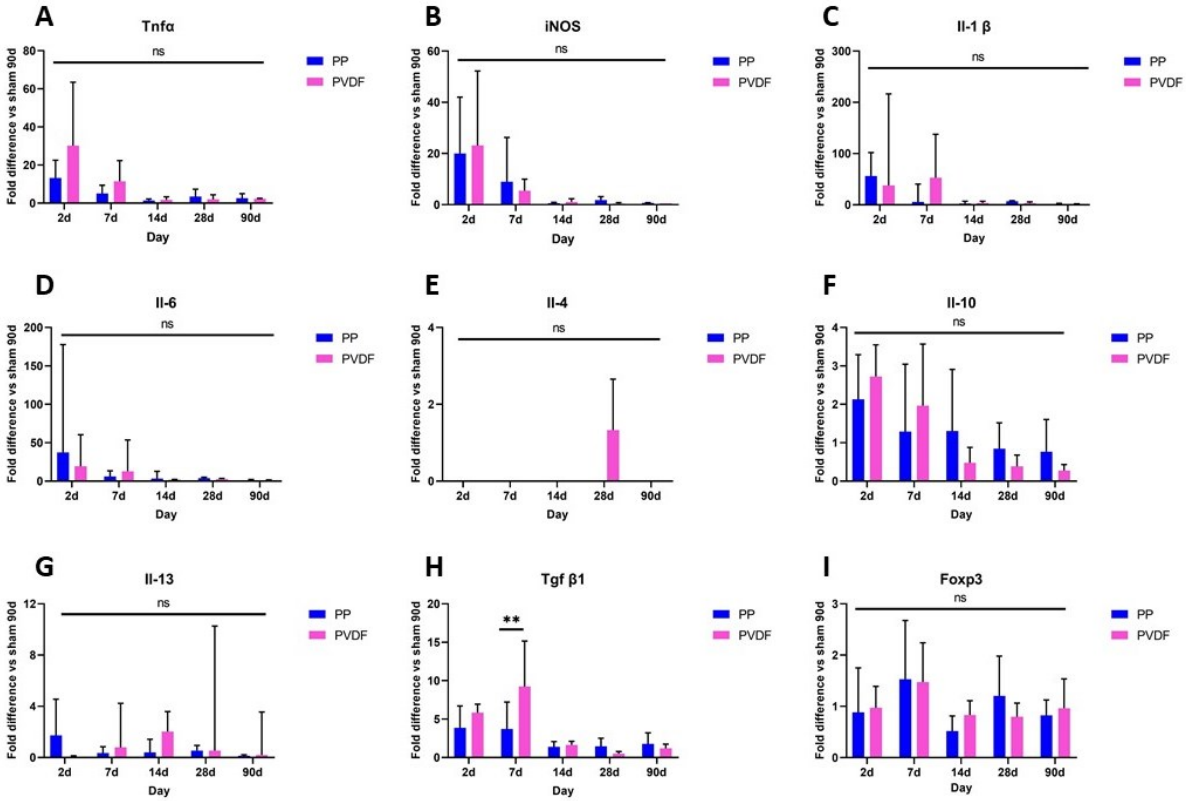

Fig. 5. Graphs displaying gene expression results: **Genes tied to type 1 macrophage phenotype:** (**A**) Tnfα, (**B**) iNOS, (**C**) Il-1β, (**D**) IL6, **Genes tied to type 2 macrophage phenotype:** (**E**) Il-4, (**F**) Il-10, (**G**) Il-13, (**H**) Tgf β1, (**I**) Foxp3. Data are shown as relative expression, normalized with sham operated animals at 90d. Data on graph a, b, f, h and i are normally distributed, N=5, displayed as mean ± SD, Two-way ANOVA or mixed effects analysis (if some values were missing); Data on graph c, d, e, and g are not normally distributed, N=5, displayed as median ± IQR, Mann-Whitney test. Asterisks mark significance * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ **** $p < 0.0001$, ns stands for not significant.

DISCUSSION:

The main objective of this study was to compare the biomechanical properties of explants with identical textile structure but made from different polymers. Preimplantation testing showed that PVDF was slightly (6,5%) stiffer. In essence, the host response was very similar as well as eventual abdominal wall compliance at different time points. The few differences in host response that were picked up on selected molecular and immunohistochemical read outs may not be relevant or even coincidence. Along the same lines, tissue integration into the host was comparable. Conversely, when comparing to SHAM operated animals, both implants induce a similar degree of muscle atrophy.

It is assumed that the host immune response and subsequent fibrotic reaction, integration and biomechanical properties of the tissues, depends on the polymer choice, the surface area in contact with the host [177, 298], and the preimplantation stiffness[299]. In this experiment, the polymer was the main variable, as the surface was kept identical and there was a minimal difference in pre-implantation stiffness. Both implants however did induce thinning of the underlaying muscle, compared to a sham control. This suggests that the presence of a implant as the ones used in this experiment, has secondary effects on the abdominal wall musculature, something we also observed with other type of implants in the same[295] and other models[300]. One mechanism that has been named to explain this, is “stress shielding”, i.e. a phenomenon that occurs when two solid materials are connected[301, 302] Following implantation, the stiffer material (in our case, the mesh) buffers or ‘shields’ the adjacent tissue (in our case, the muscle) from experiencing the physiological loads (forces) to which it is normally exposed. In the absence of loading, the less stiff tissue degenerates[303]. The somewhat higher stiffness of the mesh implant than that of native tissue, may support this mechanism. In retrospect, it is a pity that we did not include observations beyond the 180 d time point, as the process may be progressive. Given Sprague-Dawley rats may live up to 2.5-3.5y, addition of more distant time points up to one year would have been possible.

Comparison of PP and PVDF was previously done in clinical and translational studies. Klinge did a study in a rat model, comparing a heavy weight PP mesh (109 g/m²; surface: 283 mm²) to two PVDF meshes of lower weight (73 g/m² and 236 mm², resp. 45 g/m² and 119 mm²), used to overlay 2x3cm full thickness defects[296]. The endpoints were the host response (assessed by histology) and tensiometry. They concluded that PVDF implants induced a “lower fibrous tissue reaction and lower bending stiffness”. In that experiment, however, several variables were modified, i.e. the polymer, the filament size, hence the weight and also the surface. In another study, Klink implanted PP (surface: 1.1 m², pore size >2 mm, filament diameter: 140 µm) and PVDF (surface: 2.0 m²; pore size >1 mm; filament diameter 160 µm) meshes subcutaneously in rats, and measured the host response[304]. Outcomes were comparable, except for a reduction in the dimensions of the granulomatous reaction around the PVDF material, despite it having broader filaments, smaller pore size, hence larger surface area. That would suggest that, despite a higher effective surface and comparable cellular response, PVDF would induce a smaller foreign body granuloma. Our experiment however takes better control of these variables, which are named to be associated with the nature of the foreign body reaction, by keeping filament diameter, pore size and surface identical, yet only changing the weight of the implant by using another polymer. As a consequence, however, the dry properties of the visibly identical textile implants are also slightly different.

There is also some clinical literature comparing PP tot PVDF implants. Balsamo et al. retrospectively compared the surgical, anatomical, and functional outcomes of patients undergoing sacrocolpopexy with either PVDF (pore size: 1.2*1.3 mm; no weight given) or PP (pore size: 1.7 *1.7 mm; weight: 39 g/m²) mesh. They showed that patients implanted with PVDF had a comparable outcome, except for some better functional results (urinary storage symptoms and sexual function)[305]. Kavallaris et al. conducted a very similar retrospective study. Patients implanted with PP had significantly more mesh infections and exposures compared to those implanted with PVDF including more vaginal pain and discomfort[306]. No mesh characteristics were however provided. Both studies were done with commercially available PP and PVDF, which have a different textile structure, so it is difficult to say what would explain the different outcome.

This study has some weaknesses. One of them is the comprehensiveness of the used methodology. There is a huge spectrum of outcome measures available for implant studies, from which we only used a selection. Therefore we may not completely capture the host response, tissue integration and mechanical behavior. We acknowledge that other methods might have been used as well, such as biaxial mechanical testing [307] (which would better describe the mechanical characteristic in two directions); more comprehensive gene expression and gene ontology analysis[308] for more detailed view on biological processes occurring after mesh implantation; additional stainings characterizing collagen synthesis and deposition[309], nerve ingrowth[310] and neovascularization[311] for better understanding of tissue integration. However, we did not aim to apply all possible test. We believe that with the selected outcome measures we targeted the most relevant outcomes to answer our scientific questions. Another possible improvement would be done by adding later time points e.g. to study muscle degeneration. Using the rodent model has its own natural limitations and results may not be translated directly. The strengths of this study are its design including power calculation, randomization, blinding of the surgeons and outcome assessors, a targeted spectrum of outcome measures that we were familiar with, and well characterized meshes. In future work we could aim to repeat this experiment in a larger animal model, and on different implantation locations. For abdominal implantation, one could use the rabbit: they live longer, have an abdominal wall with a relatively large surface, making it possible to test two implants simultaneously within the same host. More important is that one can perform more comprehensive biomechanical testing. In rabbits, the elastance (change in intraabdominal pressure per unit change of intra-abdominal volume) of the anterior abdominal wall is rather high[161]. It may therefore be a good and sensitive model for testing the biomechanical behavior of implants[312]. Another option is using the ewe. In this large animal, both abdominal as well as vaginal implantation is possible, hence it can also serve as a model for POP surgery[190].

In conclusion, we have shown in a rat abdominal wall reconstruction model, that meshes with identical textile structure, though with made of different polymers and different weight, induce comparable compliance

MATERIAL AND METHODS:

Implants

This experiment involved two non-resorbable Amid type I macroporous implants with exactly same macrostructure with as a single difference the polymer (either polypropylene or polyvinylidene fluoride), purposely manufactured by FEG Textiltechnik GmbH (Aachen, Germany) (details in Table S1; Fig. S1). Both meshes were produced with warp knitting techniques and sterilized according to ethylene oxide sterilization method.

Animals and study design:

One hundred seventeen adult female Sprague-Dawley rats were provided by the Zootechnical Institute of the KU Leuven. The age and weight of animals were comparable (12 weeks old, 250-300 g). They were randomly assigned to one of four experimental groups, based on the closure technique used (Table S2), either coverage of

the abdominal wall defect with polyvinylidene fluoride (PVDF), polypropylene (PP), no coverage (SHAM; as positive controls), and unoperated animals (serving as negative controls) (Figure S1).

Experimental procedures:

Anesthesia and analgesia:

Anesthesia was induced in a gas chamber by inhalation 5% Isoflurane (IsoVET, 1000mg/g, Mecan VHM BV, Nijmegen, Netherlands) in 100% of oxygen (0.5 L/min), followed by intraperitoneal injection (IP) of a mixture of ketamine 80mg/kg (Anesketine, Eurovet, Heusden-Zolder, Belgium) and xylazine 5 mg/kg (Xyl-M 2%, VMDvet, Arendonk, Belgium). Postoperative animals received buprenorphine (Vetergesic, Ecuphar, Oostkamp, Belgium) 0.1 mg/kg b.i.d. for pain relief for two days.

Surgery:

First the abdominal wall was shaved and disinfected with chlorhexidine (Chlorhexidini Alcoholicus Gluconaat 0,5%, Cedium, QUALIPHAR, Bornem, Belgium). Animals were draped in a sterile manner and kept on a heating pad until recovery. Then the skin was incised right of the midline and an incisional defect of 4 cm long was made paramedian on the left (Figure S1A). The defect included the abdominal wall muscles, though the fascia transversalis and peritoneum were left intact. In the rats from the PVDF or PP groups, a 40 x 25 mm strip of mesh was laid over the defect and fixed with six interrupted 4-0 Polypropylene (Ethicon, Dilbeek, Belgium) stitches, one at each corner and one on either side halfway the long axis (Fig. S1D). In sham operated animals, no mesh was used, but the polypropylene sutures were used to mark the location of the incision (Fig. S1E). Then the subcutis was closed with a running 4-0 polyglecaprone suture (Monocryl, Ethicon), and the skin with a running 3-0 polyglactin suture (Vicryl, Ethicon). Elizabethan collars were used for 24 to 48 hours to prevent auto-mutilation. After recovery from anesthesia, rats were returned to their group cages.

Assessments

Rats were clinically examined daily during the first week postoperatively and then monthly until euthanasia, and all complications were noted. Animals with signs of auto-mutilation were reported, excluded, and replaced. On day 2, 7, 14, 28, 90 and 180 after implantation we euthanized rats (Table S2) by intracardiac administration of a mixture of embutramide, mebezonium and tetracaine hydrochloride (T61; MSD, Brussels; Belgium; 4-6 mL / 50 kg) under anesthesia with isoflurane. On obduction, gross anatomical examination included identification of fluid collections, infection, or mesh exposure, which were photographed. The former surgical area was resected 'en bloc', including the initial implant area and one cm of the neighboring native tissue, using the PP sutures as landmarks. The explant in other words included from medial to lateral, native tissue, interface with, and the implant itself with variable degrees of ingrown tissue. The explant was cut into three strips, perpendicular to the long axis of the animal (Figure S2): the upper strip for fixation in 4% paraformaldehyde, the bottom strip was stored in -20°C for later biomechanical testing and the middle part was snap frozen at -80°C for molecular testing.

Real Time Polymerase Chain Reaction (RT-PCR) analysis:

RNA was extracted using TRIPURE Isolation Reagent (Roche, Vilvoorde, Belgium) as per manufacturer's instructions. The quality of the extracted RNA was measured in a spectrophotometer at 260 nm absorbance and run on a 1% agarose gel. RNA was visualized by using GelRed (Invitrogen; Thermo Fisher Scientific, Waltham, Massachusetts, United States). 0.5 µg of total RNA was used to synthesize cDNA by using the RT2 First Strand Kit (SABiosciences, Frederick, MD, USA) based on the manufacturer's instructions. Thereafter, rat Custom RT^2 Profiler PCR Array (SABiosciences, CLAR23854) was designed for detection of a selection of genes (Table S3 and S4) that are tied to macrophage subtypes [type 1 (Tumor necrosis factor [Tnfα], inducible nitric oxide synthase [iNOS], interleukin 1 beta [Il-1 β], interleukin 6 [Il-6]) and type 2 (Interleukin 4 [Il-4], Interleukin 10 [Il-10],

interleukin 13 [Il-13], transforming growth factor beta 1 [Tgf β1]) and Treg lymphocyte (forkhead box P3 [Foxp3]), hence believed to be relevant to the host response to an implant[313]. The RT^2 Profiler PCR Array was performed according to manufacturer's instructions using the StepOnePlus Real Time PCR system (Applied Biosystems, Thermo Fisher Scientific, Waltham, Massachusetts, United States). The raw data obtained will be uploaded into StepOne software 2.3 for analysis.

Histology and immunohistochemistry:

After 24h in PAF, implants were immersed in phosphate buffered saline (PBS) for one hour, stored in 70% ethanol, embedded in paraffin, cut in five µm sections and stained with Hematoxylin & Eosin (H&E), Masson's Trichrome and immunostains for CD80, CD206, CD163, CD34, GAP42 and anti-TGFβ (Table S5). Images were captured with an Axioskope (Carl Zeiss, Oberkochen; Germany) at 25x and with an Eclipse Ci (Nikon, Melville, NY; U.S.A.) at 200x magnification. From each specimen up to ten photographs were taken randomly, depending on the specimen size. Slides were evaluated with two experienced observers who were blinded to the treatment group. For the morphometric analysis, Fiji software[286] was used. The digital color images were segmented (color deconvolution plugin) and further binarized to measure the percentage of the area stained. FBGCs were manually quantified using cell counter plug-in in H&E staining (200x magnification). Masson's Trichrome was used to semi-quantify collagen (200x magnification), muscle and fascia content (25x magnification). Vascularity was quantified by endothelial cell marker CD34 (200x magnification). CD80, CD206, CD163 staining was used to semi-quantify M1 and M2 macrophage presence (200x magnification). GAP43 stain was used at 90 days as a neuronal marker. Anti-TGFβ antibody was used at 7 days to confirm PCR findings.

Biomechanical testing:

Approximately 8.3 mm x 25mm was cut out for uniaxial tensiometry. The actual width and thickness of the samples were measured with an accuracy of 0.05 mm with analogue caliper (Richter, Speichersdorf, Germany). Measurements were taken with a caliper at three locations along the length of the sample and averaged to assess sample thickness homogeneity. The explants were tested by uniaxial tensile load testing, using a Zwick tensiometer (Zwick GmbH & Co. KG, Ulm, Germany) with a 200 N cell load and using pneumatic clamps. To avoid buckling, a preload of 0.05 N was applied using a constant elongation rate of 10mm/min. The moment preload was reached, was defined as elongation zero. Then, a constant elongation rate of 10mm/min was used to load the specimen until failure along the longitudinal axis. The test ended when the load dropped below 60% of the maximum measured value. Corresponding stress-strain curves were computed. Engineering strain was calculated by dividing the *current* clamp-to clamp distance by the clamp-to-clamp distance at *preload*. First Piola-Kirchhoff stress was calculated by dividing the current force by the initial cross-sectional area (i.e., width times thickness).

The parameters describing the mechanical properties of the native tissue and explants were obtained from the stress-strain curve. Linearized stiffness moduli were calculated for 10% strain intervals by determining the slope of a linear line fitted through the first and last point of the interval. The stiffness modulus in the comfort zone[179] was defined as the modulus noted over an interval of 10-20 % strain. The modulus in the stress zone was defined as the modulus over an interval of 70-80 %strain.

Samples size:

The primary outcome measure was the Young's modulus in the comfort zone of the explant at 90d. Power calculation was based on previous observations with polypropylene and sham operated animals on file. Using an independent t-test, 80% power, $\alpha = 0.05$ and effect size 1.31, 11 animals are needed per group and time point. For the earlier time points (<28d) dedicated to study the wound healing process, five animals were used as in previous experiment[198].

Allocating animals to experimental groups:

For allocation of study animals an online randomization tool was utilized (https://www.randomizer.org/). The animals assigned to a certain time point were operated, examined or euthanised in one batch and in random order.

Statistics:

Normality testing was done by Kolmogorov-Smirnov test. When distribution was normal, unpaired t-test (two-tailed, CI 95%) or 2 way-ANOVA (mixed effects analysis when some values were missing) with Sidak multiple comparison test was performed. When not, Mann-Whitney test or Kruskal-Wallis statistical tests with Dunn's multiple comparison test were performed. Data are presented as mean and SD or median and IQR as appropriate. Significance was reached when the condition $p < 0.05$ was fulfilled. The alpha level for all tests was 0.05. Experimental details and the statistical tests used are listed in each figure legend. All data are presented and includes all outliers. Data were processed using GraphPad Prism version 8.40 for Windows (Graph Pad Prism, La Jolla, CA, USA).

Ethical statement:

This animal experiment was approved by the Animal Ethics Committee of the KU Leuven (P059/2018). It performed in accordance with the Animals (Scientific Procedures) Act 1986 and in compliance with the EU-(Directive 2010/63/EU) and Belgian regulations (Royal Decree of 29 May 2013). Researchers followed the ARRIVE guidelines, the checklist for it can be found as supplementary material.

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We did not receive funding for this experiment. FEG Textiltechniken manufactured and provided the implants.

Author contributions:

This study was designed, and its protocol was prepared by K.M., M.G.M.C.M.C., A.M.N.M., R.D., L.H.H., H.F., N.F., L.K. and J.D. Surgeries, clinical examinations and obductions were performed by K.M., M.G.M.C.M.D.C., A.M.M. and R.D. Histology and immunohistochemistry was performed by K.M., M.G.M.C.M.C., A.M.N.M., R.D., L.H.H., biomechanical testing was performed by K.M. and H.F. and N.F., Analysis of data and statistical analyses were performed by K.M., M.G.M.C.M.C., L.H.H., H.F., N.F., assisted by L.K., E.M. and J.D. The original manuscript was drafted by K.M. under supervision of J.D. All authors critically reviewed and revised the manuscript and approved the final version.

Competing interests: JDP holds a generic research grant ("Leerstoel") by DUO-MED, entitled "Pelvic Organ Prolapse – Advanced Research and Technology", managed by the transfer office of the KU Leuven. All research is done independently from the funders; i.e. they do not have any control over the design, execution or reporting of the results.

Data and materials availability: All data are available on demand via corresponding author.

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SUPPLEMENT:

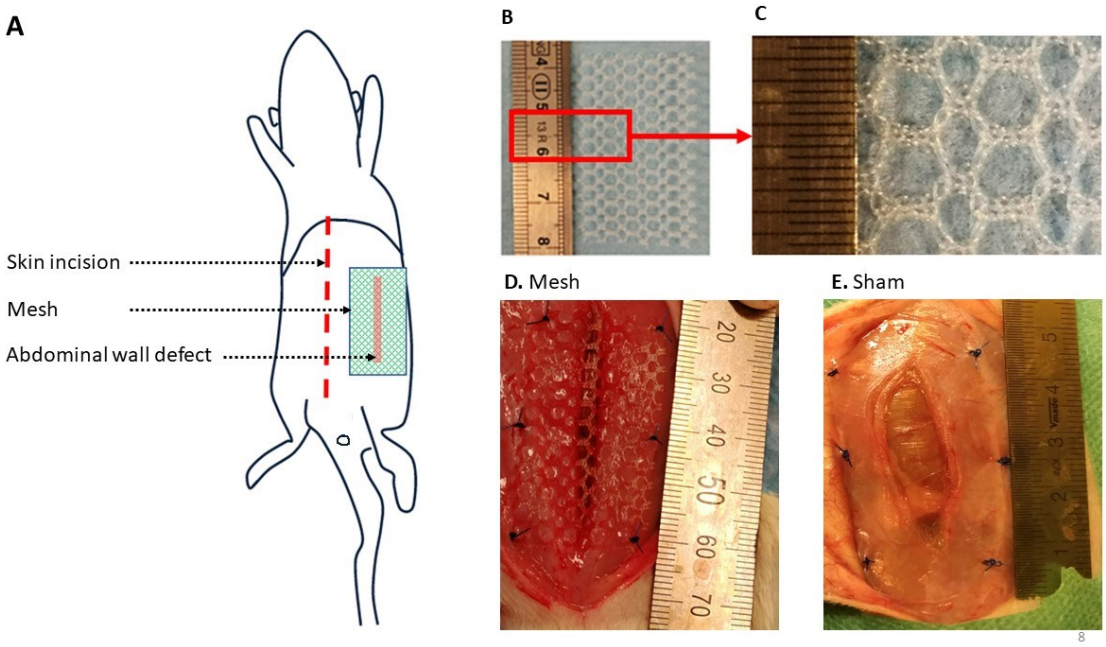

Fig. S1. Mesh implantation. **(A)** Schematic representation of the surgery **(B)** Mesh implant **(C)** Detail of the mesh structure **(D)** Representative photography of the Implanted mesh **(E)** Representative photography of the sham surgery with (blue) polypropylene stitches demarking surgery area

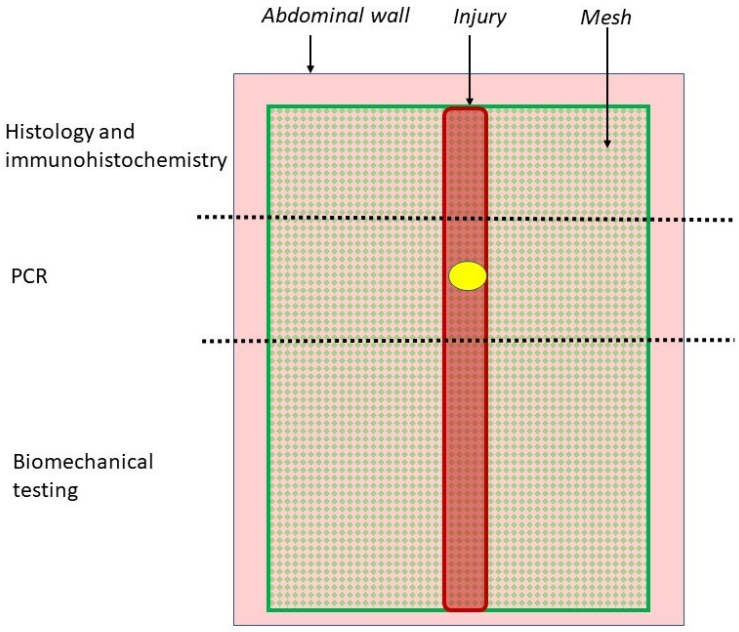

Fig. S2. Schematic representation of the explant division. Yellow dot represents the tissue for PCR.

Table S1: Characteristics of implants.

Implant	PP	PVDF
Molecular weight polymer [g/mol]	42	64
Size [cm]	4 x 2.5	4 x 2.5
Pore size [mm]	2 x 2.2 x 3	2 x 2.2 x 3

Textile porosity [%]	66	66
Effective porosity [%]	61	61
Classification	Medium - weight	Heavy - weight
Thickness [mm]	0.78	0.78
Weight [g/m²]	64	125
Young's modulus [N/mm²] mean ± SD	6,604 ± 0,3005	7,049 ± 0,3902

Table S2: Division of animals to treatment groups and time points.

Treatment group/time point		**2**	**7**	**14**	**28**	**90**	**180**
PP		5	5	5	5	11	11
PVDF		5	5	5	5	11	11
SHAM						11	11
Not operated	11						

Table S3: List of genes and stainings used in this experiment and their function

Real Time Polymerase Chain Reaction (RT-PCR)		
Protein	**Gene**	**Gene function**
TNFα	Tnfα	Genes tied to M1 macrophage phenotype
iNOS	iNOS	
IL1β	Il-1 β	
IL6	Il-6	
IL4	Il-4	Genes tied to M2 macrophage phenotype
IL10	Il-10	
IL13	Il-13	
TGFβ1	Tgf β1	
FOXP3	Foxp3	Genes tied to Treg lymphocyte
Histology		
Staining		**Marker for**
HE		Foreign body giant cells
Masson trichrome		Fibrous tissue around fibres Muscle and fascia content
Immunohistochemistry		
epitope		**Marker for**
CD34		Endothelial cells (vascularization)
CD80		M1 macrophage phenotype
CD163		M2 macrophage phenotype
CD206		M2 macrophage phenotype
GAP43		Neuronal marker (axonal growth and regeneration)
TGFβ1		TGF β1

Table S4: List of primers used

Genes	**Forward**	**Reverse**
PGK	ATGCAAAGACTGGCCAAGCTAC	AGCCACAGCCTCAGCATATTTC
GAPDH	CAACTCCCTCAAGATTGTCAGCAA	GGCATGGACTGTGGTCATGA
eGFP	CATGGTCCTGCTGGAGTTCGTG	CGTCGCCGTCCAGCTCGACCAG

Table S5: List of antibodies used

Protein	**Concentration**	**Catalogue number**	**Brand**	**Country**
CD34	1/1000	ab81289	Abcam	Cambridge, MA, USA
CD80	1/100	sc-9091	Santa Cruz Biotechnology	Dallas, TX, USA
CD163	1/300	MCA342R	Bio-Rad	Hercules, CA, USA
CD206	1/300	sc-34577	Santa Cruz Biotechnology	Dallas, TX, USA
GAP43	1/250	ab75810	Abcam	Cambridge, MA, USA
TGF β1	1/500	ab215715	Abcam	Cambridge, MA, USA

Chapter 8

General discussion and perspectives

GENERAL DISCUSSION AND PERSPECTIVES:

Pelvic floor dysfunctions and genitourinary syndrome of menopause are very common in women and may significantly decrease their quality of life[314, 315]. As the population is aging, increasingly more women are and will be affected[314]. Traditional treatment modalities such as surgery for symptomatic POP, systemic or local hormonal replacement for GSM, may not be sufficient or safe for everyone. Some patients also seek for alternatives, because they are afraid of the invasiveness or possible side effects of surgery or use of hormones. Therefore, novel treatment modalities are still being developed and often offered prior to proper evaluation. The overall aim of my thesis was to experimentally assess a selection of such novel treatment modalities for PFD and GSM.

My research started with an extensive literature search, prior to purpose designed animal experiments. As a translational research group, we have previously used a wide spectrum of different animal models starting with mouse[316], rat[297], over rabbits[300] to end up with a larger animal model as the ewe[317]. We first aimed to summarize the current knowledge on animal models of the pathogenesis and treatment of selected PFD, i.e. POP, and surgery using mesh (**Chapter 2 and 3**).

In chapter 2 we summarized all experimental studies on animal models for POP done during the last 20 years, and the utility these models may have for investigating the pathophysiology and novel therapies of POP[318]. We systematically searched and identified 7426 articles, from which 51 fulfilled inclusion criteria. We are not aware of a similar work of that extensity done before. Our systematic review revealed not only the details on individual models, their advantages, and disadvantages, but also identified methodological shortcomings of experiments with animals dedicated to POP. The analysis substantially helped us to design our later animal experiments. Apart from that, the conclusion of that systematic review was that several species are being used as a model of POP, each of them with different purposes. From all species used, only one non-human primate (the squirrel monkey) develops POP spontaneously. However, use of primates is controversial and de facto forbidden in our country. In our research we therefore chose rat for simulated POP repair with synthetic mesh (**Chapter 7**) and sheep for a study on the use of LASER therapy for simulated GSM (**Chapter 6**).

In Chapter 3 we reviewed the literature describing the use of animal models for testing already used and new materials being proposed for the surgical treatment of pelvic organ prolapse[312]. Synthetic implants used for vaginal prolapse reconstruction, were introduced clinically without proper translational research supporting their use. They were aggressively marketed, and their use became embraced, but soon adverse events surfaced, leading to reinterventions or chronic pain, suffering of patients, and logically also several lawsuits. Meanwhile most vaginal implants for prolapse surgery have been withdrawn from the market or even forbidden in many countries[319]. In some countries there is even a ban on the use of smaller tapes for the treatment of urinary incontinence[320].This leaves a selection of patients without an efficient treatment option. Translational research into novel, safe and effective implants is encouraged by the same international authorities, who warned against the use of currently available products[61]. Unfortunately, our review shows that, although there is an abundance of animal models, there is a lack of standardization in methodology. Apart from that, that review together with the review presented in **Chapter 2**, made us conclude that the rat model seemed the best first fit for our future work with candidate pelvic floor implants (**Chapter 7**).

In the **Chapter 7** we tested such a material for pelvic floor surgery as an alternative for polypropylene (PP), i.e. a polyvinylidene fluoride (PVDF). In a unique and original study, we compared structurally identical PVDF and PP in the rat incisional abdominal hernia model. Main outcome measure was the biomechanical behaviour of explants; additional measures were the host inflammatory response and tissue integration. We did not observe differences in biomechanical properties between the two materials, also differences in host response and tissue integration were minimal, if at all relevant. Both implants induce on the longer term (PP from 90 d onwards; PVDF at 180 d) limited muscle degeneration. This observation is in line with previous findings in a rabbit model[300]. To our knowledge this is the first experiment comparing two different polymers that have *exactly the same textile*

structure. Previous translational and clinical comparisons of PP and PVDF[296, 304-306] were done with different mesh weight and knitting patterns making the comparison of the polymers or materials itself impossible. Recently, there was a study into the resistance to degradation suggesting that PVDF is more resistant to biodegradation compared to PP under SEM. On ultra-structural evaluation, "cracking, flaking and peeling" was documented. The clinical relevance of this is unclear, and the claim that PP "cracks" was actually doubted in other studies[321], and anyway the implants used in the former experiments, had a different textile structure, so that it remains unclear that the polymer itself is too blame[322]. It remains therefore uncertain whether the polymer PVDF has inherent advantages that would justify its use, in view of its more expensive purchase cost in many countries (e.g. up to 18 times in Germany according to an abstract)[323].

In another animal study (**Chapter 6**), we simulated Er: YAG LASER treatment for GSM, for which we choose the sheep as model (**Chapter 2**). To design that experiment we first reviewed the literature on the measurable effects of non-ablative Er:YAG LASER on the skin and vaginal wall in **Chapter 4**[324]. In that systematic review, we included 15 out of 7187 identified papers. In only four studies, Er:YAG LASER was applied on vaginal tissue. Again, that low number is contrasting with the already widespread use of LASER in clinical practise. Er:YAG LASER energy induces measurable changes in the deeper skin or vaginal wall by a process of cell activation, stimulation of production of extracellular matrix and tissue remodelling. However, the level of evidence was low, and the literature is seriously biased, when assessed by the Robins I tool. Also, the range of outcome measures was wide and heterogenous. Out of these we carefully chose the outcome measures and methods used in the experiment described in **Chapter 6**.

In that chapter we describe a randomised controlled study to measure the effects of non-ablative Er:YAG LASER, compared to sham and oestrogen controls, on vaginal atrophy following surgically induced menopause in the ewe[325]. This experiment was based on a pilot study in which Er:YAG was first used in sheep by one of my predecessors[46]. That earlier experiment demonstrated that ovariectomy does induce menopausal changes, and that laser application had only limited effects on the short term (7 days). In my experiment, the observation period was longer, positive controls were added, and we waited for six months after ovariectomy, so that ewes would have for certain full or maximal signs of menopause and vaginal atrophy, which we now also documented by serial biopsies. Again, we demonstrated that LASER application induces comparable changes to the vaginal epithelial thickness as well as vaginal compliance as those observed after SHAM vaginal manipulation. The effects are also less than what is observed after systemic oestrogen replacement.

This work triggered a lot of correspondence. First, there were questions about the use of ewes, which they found not to be comparable to the human vagina[326, 327]. The ewe however was earlier repeatedly used as a model of menopause, both by our and other groups, and to our opinion it is the closest animal model to humans apart of nonhuman primates[46, 262, 265, 318]. Further, translational research remains important because one can test a wide spectrum of outcome measures which would not be acceptable in human subjects. Other argued[328] that also CO_2 laser should have been used. Actually, we did a similar parallel experiment, which was led by a colleague doctoral student, where we used the Mona Lisa Touch hardware. Although that work is not published yet, preliminary findings were presented at the IUGA Annual Meeting 2020[329], showed similar results to that of the first study; CO2 LASER indeed had no significant effect on vaginal epithelial thickness compared to sham applications, whereas local oestrogen did have. To our knowledge there is no additional translational or clinical work assessing *Er: YAG laser* published from the time of publishing our study. Meanwhile we have embarked on to a well powered randomised sham-controlled clinical trial (clinical trial gov number: NCT04021966) to assess the effect of CO2 LASER for the treatment of GSM. Our first yet unpublished study results confirm that also clinically, LASER performed only as good as placebo. In that study no oestrogen supplementation arm was included.

In Chapter 5 we conducted another systematic review on LASER therapy for POP and UI[330], conditions for which the use of LASER is also claimed. We included 31 studies with data on 1530 women and identified only one randomised controlled trial[214] and two controlled cohort studies[222, 223]. All studies reported an improvement of

POP or UI after LASER, but the quality of studies was mostly poor. The risk of bias in the RCT was low; conversely the controlled studies had a serious risk of bias. We also observed a wide heterogeneity of LASER settings, application protocols and outcome measures. LASER meanwhile has become extremely popular amongst gynaecologists and patients for its easy and mostly painless application, which takes only minutes. Also, it does not require complex training, health examination or admission. Also, in the world celebrating never-ending youth, rejuvenation of vagina or reversal of incontinence sells very well. Therefore, we have embarked on two randomized clinical trials, one in incontinence and one in prolapse patients (clinical trial gov numbers: NCT04523298, NCT04643353).

Future perspectives:

In conclusion there is a need for better quality experimental and clinical studies and hard evidence for interventions that are frequently done, or are being first introduced, in urogynaecology patients. Those studies available have one or more methodologic shortcomings and bias is mostly serious. Future translational studies should include animals that have comparable baseline characteristics such as weight, sex or age. Furthermore, blinding of the researchers taking care of animals as well as researchers analysing the outcomes is needed to avoid bias. Also, the methodology section should be better described to avoid misinterpretation and allow to repeat the experiments by others. Regarding the heterogeneity of the outcome measures in translational studies, one potential solution is the development of core outcome sets as the Comet Initiative developed in clinical trials (https://www.comet-initiative.org/).

As to further mesh testing, we suggest that our implantation study should be expanded to a larger animal model and/or insertion of the same implants on different locations. In sheep, both vaginal and abdominal implantation may be considered, as well as performing sacrocolpopexy[331]. For abdominal wall implantation in large animals, the rabbit may work as well. They live longer and they have an abdominal wall with a relatively large surface, making it possible to test two implants simultaneously within the same animal, as we previously did[198]. In both larger species, more comprehensive biaxial biomechanical testing is possible as well, which could disclose different outcomes that were not picked up in the rat experiment we did. However, at this time we would be inclined to speculate that PVDF and PP mesh would behave similar in a clinical application.

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Chapter 9

Summary, samenvatting, souhrn

SUMMARY:

We aimed to test selected novel treatment modalities for pelvic floor dysfunctions and genitourinary syndrome of menopause. Both conditions are common in female, they negatively affect their quality of life, and current treatment options are not optimal.

We started our research with an extensive literature search. First, we summarized the information on animal models for, and the utility they may have in the investigation of the pathophysiology of POP and novel therapies. We systematically searched 7426 articles from which 51 fulfilled the inclusion criteria. From all screened animals, only the non-human primate develops POP spontaneously, however their use is controversial. We concluded that many studies have methodological shortcomings and lack standardization in reporting outcomes. Also, several other animals can be used as a model of surgery for POP, each of them with different purposes. For our later research we chose the rat model to simulate POP repair with synthetic mesh.

We also systematically reviewed the literature on the objective effects of non-ablative Er:YAG LASER on the skin and vaginal wall. We identified 7187 articles of which we included 15 in our review, including four that tested Er:YAG LASER on vaginal tissue. Er:YAG LASER energy induces measurable changes in the deeper skin or vaginal wall by a process of cell activation, production of extracellular matrix and tissue remodelling, however the evidence level was low with serious risk of bias in most articles, and a wide spectrum of outcome measures. This review helped us to build the design of an animal study. We also reviewed the literature on LASER therapy for POP and UI. We included 31 studies on 1530 adult women. All studies reported an improvement of POP or UI after LASER use, but the quality of studies was mostly poor and risk of bias serious. We identified only one randomised controlled trial and two controlled cohort studies in urinary incontinence and using standardized validated tools. The risk of bias in the RCT was low; the controlled studies had a serious risk for bias. Unfortunately, there was a wide heterogeneity of LASER settings, application protocols and outcome measures. That review helped our group designing three RCT, one in GSM, one in POP and one for urinary incontinence; work that will be reported by one of my successors.

In the experimental part of my thesis, I conducted two animal translational studies. In the first we did a randomised controlled trial aiming to measure effects of non-ablative Er:YAG LASER on vaginal atrophy in the ewe menopausal model, as compared to sham and oestrogen application. We demonstrated that both the vaginal epithelial thickness as well as the vaginal compliance were modified by LASER and SHAM manipulation to a similar extent, but less than what was observed following systemic oestrogen replacement.

In the second experiment we preclinically tested a polyvinylidene fluoride (PVDF) mesh, used for POP surgical repair. We implanted the material in the rat incisional abdominal hernia model. We compared outcomes to those obtained after implantation of a structurally identical mesh but made from polypropylene (PP). Main outcome measure was biomechanical behaviour of explants, next to host inflammatory response and tissue integration. Biomechanical testing showed no difference between the two materials. Also, the host response and tissue integration were almost identical, and both implants caused ultimately some degree of muscle atrophy in later time points. In conclusion, we first demonstrate there is no difference in host response to implants either made from PP or PVDF when they have the same textile properties.

In conclusion, treatment of GSM, POP, and UI with Er: YAG laser is not supported by good quality evidence. Second, in the ovine menopause model laser therapy has an effect that is no different from that of sham manipulation, and both have less effects than systemic oestrogens. Third, in the rat model, implants that have an identical textile structure but that are made from a different polymer (PVDF or PP), hence have a different weight, generate the same biomechanical properties, host response and tissue integration. Both induce muscle atrophy on the medium term.

SAMENVATTING:

In dit werk werden nieuwe behandelingswijzen voor bekkenbodemdisfuncties en het urogenitaal syndroom van de menopauze gestest. Beide aandoeningen komen vaak voor bij vrouwen, hebben een negatieve invloed op hun kwaliteit van leven en de huidige behandelingsopties zijn niet optimaal.

We begonnen met een uitgebreid literatuuronderzoek. Eerst vatten we de literatuur samen over diermodellen gebruikt in studie van de pathofysiologie van prolaps (POP) en nieuwe behandelwijzen. We doorzochten systematisch 7426 artikelen waarvan 51 voldeden aan de inclusiecriteria. Van alle genoemde diermodellen ontwikkelen alleen de niet-humane primaat spontaan POP, maar het gebruik ervan is controversieel. We concludeerden verder dat veel studies methodologische tekortkomingen hebben en dat er geen standaardisatie van de rapportering is. Verschillende diersoorten kunnen worden gebruikt als model voor chirurgisch herstel van POP, elk met hun eigen voor- en nadelen. Voor ons verder onderzoek naar de weefselrespons naar synthetische mesh kozen we het rat-model.

We voerden ook een systematisch literatuur onderzoek uit naar de objectieve effecten van niet-ablatieve Er:YAG LASER op de huid en vaginale wand. We identificeerden 7187 artikelen en namen er 15 op in onze review, inclusief vier studies waarin Er:YAG LASER werd getest op vaginaal weefsel. Er:YAG LASER-energie induceert meetbare veranderingen in de diepere huid of vaginale wand, en dit door een proces van celactivering, productie van extracellulaire matrix en weefselremodellering. Het bewijsniveau was eerder laag, met een ernstig risico op vooringenomenheid in de meeste artikelen, en een breed spectrum van uitkomstmaten. Deze review heeft ons geholpen bij het opstellen van een eigen experimentele studie. We beoordeelden ook de literatuur over LASER-therapie voor POP en UI beoordeeld. We hebben 31 onderzoeken bij 1530 volwassen vrouwen geïncludeerd. Alle studies rapporteerden een verbetering van POP of urinaire incontinentie (UI) na gebruik van LASER, maar de kwaliteit van de studies was meestal slecht en het risico op bias ernstig. We identificeerden slechts één gerandomiseerde studie en twee gecontroleerde cohortstudies naar UI waarbij ook gebruik gemaakt werd van gestandaardiseerde én gevalideerde hulpmiddelen. Het risico op bias in de RCT was laag; de gecontroleerde onderzoeken hadden een ernstig risico op vooringenomenheid. Helaas was er ook een grote heterogeniteit van de LASER-instellingen, toepassings-protocollen en uitkomstmaten. Dit onderzoek hielp onze groep bij het ontwerpen van drie gerandomiseerde studies, één bij patiënten met GSM, één met POP en één voor UI; allemaal werk dat door een van mijn opvolgers zal worden gerapporteerd.

In het experimentele deel van mijn proefschrift heb ik twee translationele studies uitgevoerd. We voerden eerst een gerandomiseerde studie uit om de effecten van niet-ablatieve Er:YAG LASER op vaginale atrofie bij het menopausale schaap, in vergelijking met placebo- en systemische oestrogeentoediening. We toonden aan dat zowel de vaginale epitheeldikte als de vaginale compliantie in vergelijkbare mate werden gewijzigd door LASER- en SHAM-manipulatie, maar minder dan wat werd waargenomen na systemische oestrogeentoediening.

In het tweede experiment testten we preklinisch een polyvinylideenfluoride (PVDF) matje, klinisch gebruikt voor herstel van prolaps. We implanteerden het materiaal in het buikwand-incisiemodel in de rat. We vergeleken de uitkomsten met die na implantatie van een structureel identiek matje, maar gemaakt van polypropyleen (PP). De belangrijkste uitkomstmaat waren de biomechanische eigenschappen van explantaten, naast de ontstekingsreactie van, en weefselintegratie in de gastheer. Er waren geen biomechanische verschillen tussen de twee materialen. Ook waren de gastheer-respons en weefselintegratie bijna identiek, en beide implantaten veroorzaakten uiteindelijk enige mate van spieratrofie. Wij toonden m.a.w. voor het eerst aan dat er geen verschil is in de respons van de gastheer wanneer matjes met vergelijkbare structuur worden gebruikt, ongeacht het polymeer dat ervoor wordt gebruikt.

Samenvattend kan worden gesteld dat er weinig hard bewijs is voor de behandeling van GSM, POP en UI met Er:YAG-laser. Bij menopausale schapen heeft lasertherapie een effect dat niet verschillend is van schijnmanipulatie, en beide hebben minder effecten dan systemische oestrogenen. In het rattenmodel toonden we aan dat de respons afhankelijk is van de textiel structuur, en niet het polymeer.

SOUHRN:

Naším cílem bylo testování vybraných nových léčebných modalit pro dysfunkci pánevního dna a genitourinární syndrom menopauzy. Oba stavy jsou u žen běžné a negativně ovlivňují kvalitu jejich života. Současné možnosti léčby však nejsou optimální.

Náš výzkum jsme zahájili rozsáhlým studiem literatury. Nejprve jsme shrnuli informace o zvířecích modelech a jejich vlastnostech pro zkoumání patofyziologie POP a nových terapií. Systematicky jsme prohledali 7426 článků, z nichž 51 splnilo kritéria pro zařazení. Ze všech zkoumaných zvířat se spontánně vyvine POP pouze u primátů, jejich použití je však kontroverzní. Došli jsme k závěru, že mnoho studií má metodologické nedostatky a postrádá standardizaci, ale že existuje několik zvířat, která lze použít jako chirurgický model pro POP, každé z nich je vhodně pro jiné účely. Pro náš pozdější výzkum jsme vybrali krysu pro opravu POP syntetickou síťkou.

Systematicky jsme také prozkoumali literaturu o objektivních účincích neablativního Er: YAG LASER na kůži a vaginální stěnu. Identifikovali jsme 7187 článků a 15 zahrnuli do našeho přehledu, z nichž pouze 4 testovaly Er: YAG LASER na vaginální tkáni. Energie Er: YAG LASERU indukuje měřitelné změny v hlubších vrstvách kůže nebo vaginální stěny procesem buněčné aktivace, produkce extracelulární matrix a remodelace tkáně, nicméně úroveň důkazů byla nízká s vážným rizikem bias ve většině článků a širokým spektrem měřených parametrů. Tato review nám pomohla navrhnout design pozdější studie na zvířatech. Prozkoumali jsme také literaturu o LASER terapii pro POP a UI. Zahrnuli jsme 31 studií na 1530 dospělých ženách. Všechny studie uváděly zlepšení POP nebo UI po použití LASERU, ale kvalita studií byla většinou špatná a riziko bias vážné. Identifikovali jsme pouze jednu randomizovanou kontrolovanou studii a dvě kontrolované kohortové studie o účinku LASERU na urinální inkontinenci, které užily standardizované validované nástroje. Riziko bias v RCT bylo nízké; kontrolované studie měly vážné riziko bias. Bohužel i zde byla široká heterogenita nastavení LASERU, aplikačních protokolů a zkoumaných parametrů. Tato review pomohla naší skupině navrhnout tři RCT, jednu pro GSM, jednu pro POP a jednu pro inkontinenci moči; Těmito studiemi se nyní zabývají mí mladší kolegové.

V experimentální části své práce jsem provedla dvě translační studie na zvířatech. V první jsme provedli randomizovanou kontrolovanou studii zaměřenou na měření účinků neablativního Er: YAG LASER na vaginální atrofii v ovčím menopauzálním modelu. Er:YAG LASER jsme srovnali s vaginální manipulací a systémovým podáním estrogenů. Ukázali jsme, že jak vaginální epiteliální tloušťka, tak vaginální poddajnost byly modifikovány pomocí LASERU a vaginální manipulace v podobném rozsahu. Tato modifikace byla menší než bylo pozorováno u zvířat po systémové náhradě estrogenu.

V druhém experimentu jsme preklinicky testovali síťku z polyvinylidenfluoridu (PVDF), používanou pro chirurgickou opravu POP. Materiál jsme implantovali do incizionálního krysího modelu břišní kýly. Porovnali jsme výsledky s těmi, které byly získány po implantaci strukturálně identické sítky vyrobené z polypropylenu (PP). Hlavním výstupním měřením bylo biomechanické chování explantátů, dále jsme zkoumali zánětlivou reakci hostitele a integraci síťky do tkáně hostitele. Biomechanické testování neprokázalo žádný rozdíl mezi těmito dvěma materiály. Také reakce hostitele a integrace byly téměř totožné. Vedlejším pozorováním je, že oba implantáty způsobily určitý stupeň svalové atrofie v pozdějších časových bodech.

Závěrem lze říci, že léčba GSM, POP a UI Er: YAG laserem není dostatečně podložena kvalitními důkazy. Za druhé, v ovčím modelu menopauzy nemá laserová terapie jiný účinek než vaginální manipulace a obě terapie mají menší účinek než systémové estrogeny. Za třetí, u krysího modelu implantáty, které mají stejnou textilní strukturu, ale jsou vyrobeny z jiného polymeru (PVDF nebo PP), a proto mají jinou hmotnost, vytvářejí stejné biomechanické vlastnosti, odezvu hostitele a integraci tkáně. Oba ve střednědobém horizontu vyvolávají svalovou atrofii.

Chapter 10

Curriculum vitae

CURRICULUM VITAE:

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Date of birth: 07 December 1988
Nationality: Czech

Education:
2017 – present PhD in Biomedical Sciences, KU Leuven, Doctoral school of biomedical sciences, Mechanism of human disease. Joint degree with Charles University in Prague, Preventive Medicine. Belgium and Czechia
2008 – 2014 MD Study Program, 2nd Faculty of Medicine, Charles University in Prague. Czechia

Professional experience:
2014 – present Institute for the Care of Mother and Child, Prague, Czechia.
Position: medical doctor, residency in specialization program: Obstetrics and gynaecology.

Skills training and development:
2017 Course on Laboratory Animal Science, KU Leuven, Belgium
2017 Creating effective research poster, KU Leuven, Belgium.
2017 Scientific integrity I, KU Leuven, Belgium
2018 Writing skills for biomedical researchers, KU Leuven, Belgium.
2018 Management of references and publishing, KU Leuven, Belgium.
2018 Getting the message across, KU Leuven, Belgium
2018 Stress management, KU Leuven, Belgium.
2018 Scientific writing for biomedical researchers, KU Leuven, Belgium.
2019 Presentation skills, KU Leuven, Belgium.
2020 Scientific integrity II, KU Leuven, Belgium.
2020 Networking, KU Leuven, Belgium.
2020 Delivering your presentation remotely, KU Leuven, Belgium.

Supervision undergraduate students:
2018 Master student - Rebecca Degliuomini, San Raffael University, Milan, Italy.
2019 Master student - Anna Marie Nora Mazzer, San Raffael University, Milan, Italy.

Conferences and presentations:
Local (Czech Republic) conferences:

2016 Usti nad Labem, XXXIII. National Conference of Perinatology and Fetomaternal Medicine, Occurrence of SGA in Women Over 40 Years of Age. Oral presentation.

2016 Valec, Conference of Hospital Gynaecologist and Obstetricians, Occurrence of Selected Complications in Mothers over 40 Years of Age. Oral presentations.

2017 Karlovy Vary, XXXIV. National Conference of Perinatology and Fetomaternal Medicine, Preeclamptic Index and its Importance in Predicting Preeclampsia and Other Pregnancy Complications. Oral presentation.

2017 Liberec, Symposium of Northbohemian Gynecologists, The Importance of the Preeclamptic Index in Management of Patients with Gestational hypertension. Oral presentation.

2017 Brno, National Conference - Section of Ultrasonic Diagnostics ČGPS ČLS JEP, Multiple Pregnancy in ÚPMD, 2012-2016; Ultrasound prediction of outcome in fetuses with left side diaphragmatic hernia. Two oral presentations.

2020 Brno, National Conference - Section of Ultrasonic Diagnostics ČGPS ČLS JEP, Ultrasound visualisation of anal sphincter and interpretation of abnormal findings. Oral presentation.

2021 Liberec, Conference of Hospital Gynaecologist and Obstetricians, Laser in urogynecology, Oral presentations.

2021 Hradec Kralove, 18th International Medical Postgraduate Conference, Vaginal Er:YAG LASER application in the menopausal ewe model: a randomized estrogen and sham controlled trial, Oral presentations.

2021 Prague, Czech Urogynecology, Vaginal Er:YAG LASER application in the menopausal ewe model: a randomized estrogen and sham controlled trial, Oral presentations.

International conferences:

2016 Rome, Italy, 26. World Congress on Ultrasound in Obstetrics and Gynaecology, Retrospective analysis of twin pregnancies in the institute for the care of mother and child between 2012 – 2015. Poster.

2017 Ljubljana, Slovenia, 16th World Congress in Fetal Medicine, sFlt-1/PlGF ratio in clinical practice: Reassessment of cut-off values for predicting preeclampsia in our cohort of patients. Poster.

2017 Viena, Austria, 27. World Congress on Ultrasound in Obstetrics and Gynecology, sFlt-1/PlGF ratio in clinical practice: Reassessment of cut-off values for predicting preeclampsia in our cohort of patients. Poster.

2019 Nashville, TN, USA, AUGS/IUGA Meeting - IUGA 44th Annual Meeting, The Effect of Er:YAG laser on the skin and vaginal wall: a systematic review. Poster.

2019 Tel-Aviv, Izrael, 12th EUGA annual meeting, Does the polymer matter? In vivo comparison of two otherwise identical textile meshes; Role of neotissue compliance on muscle atrophy following abdominal wall hernia repair in rat model. Two oral presentations.

2020 Virtual 45th IUGA Meeting. Vaginal Er:YAG LASER application in the menopausal ewe model: a randomized estrogen and sham controlled trial. Oral presentation. Winner of the best basic science abstract award.

Publications

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