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CHAPTER 8

GENERAL DISCUSSION

Pelvic organ prolapse (POP) remains a great therapeutic challenge to urogynaecologists, gynaecologists and urologists as there is no optimal treatment. Moreover, due to the multifactorial nature of the disease, and its late on-set in life, POP has been difficult to study and its aetiology remains unclear. The aim of this thesis was to gain insight into the pathophysiology of POP by identifying differences between cell behaviour, matrix composition, and cell-matrix interactions in tissues derived from women with and without POP. It was hypothesized that most patients with prolapse acquire defects on fibroblast behaviour and extracellular matrix composition. To test this hypothesis a multi-parameter study approach was used which resulted in novel findings which will be discussed in this chapter.

Acquired defects in prolapsed tissues

Women with pelvic organ prolapse have weakened tissues with altered extracellular matrix composition (ECM)¹⁻¹¹ and mechanical properties^{9,12-14}. Until starting the research presented in this thesis, only one study had shown that myofibroblasts from postmenopausal women had lower contractile capacities than cells from non-prolapsed women, indicating that the cells involved in tissue maintenance and remodelling might also be affected by prolapse¹⁵. Previous studies were designed to compare tissues from women with and without POP so it was not possible to know if the observed defects were intrinsic or acquired. In **chapters 3 to 5** of this thesis, cells and tissues were obtained from a very strict patient cohort including only Caucasian, Dutch, premenopausal women, with and without cystocele, in order to study the effect of POP while ruling out the effects of ageing. Furthermore, to distinguish between acquired and intrinsic defects, biopsies from prolapsed and non-prolapsed tissues within the same women were also included, thus each patient was her own control. Cells isolated from these tissues were evaluated *in vitro*. Cellular mechanoresponses on two different surface substrates were studied in a dynamic model with continuous loading, mimicking respiration, as presented in **chapter 2**. Fibroblastic cells' functional characteristics were evaluated in **chapter 3** in a larger cohort, using the model from **chapter 2** and a contractility assay. The combined results from **chapters 2 and 3** showed that cells derived from women with pelvic organ prolapse have less contractile capacity and lower mechanoresponses than cells derived from non-prolapsed tissues, especially on uncoated plates. Moreover, since no differences were found in any of the evaluated functional characteristics between cells derived from non-prolapsed tissues in women with and without prolapse, these defects seem to be acquired rather than intrinsic.

In **chapter 4** we also found acquired defects in the matrix composition of prolapsed tissues compared to non-prolapsed tissues. Tissue biopsies from the same patient cohort used in **chapter 3** were compared histologically and biochemically and, as with the cellular functionalities, no differences were found between non-prolapsed tissues in women with and without prolapse. However, increased pyridinoline collagen cross-links, higher

quantities of smooth muscle cells, and a tendency towards a higher content of collagen III and elastin were found in the prolapsed tissues compared to non-prolapsed tissues within the same patient. Since most patients with prolapse included in these studies seem to have acquired defects in both cell behaviour and ECM composition, a microarray analysis was used in **chapter 5** to identify specific biological molecular pathways related to POP. A cluster analysis revealed that most of the genes that had a dysregulation were clustered in two molecular pathways: (i) “the ECM/integrin pathway cluster”, and (ii) “the muscle cell/contraction pathway cluster”. These results provide evidence for inter-individual differences in non-prolapsed and prolapsed tissues, implying different possible failure mechanisms leading to POP.

Cell-matrix interactions in POP

In the previous section, differences in cell behaviour and tissue composition were established between prolapse and non-prolapse patients. Two possible molecular pathways were identified that could have led to the progression of POP in different ways in women with prolapse. However we still do not know the effect of prolapse on vaginal fibroblasts, matrix production and remodelling, nor the effect of prolapsed matrices on cell behaviour. This information would give us an idea about how cells and the extracellular matrix interact in women with prolapse. Since most patients with prolapse are postmenopausal women, the effects of POP and menopausal status on the capacity of vaginal fibroblasts to produce and remodel matrix *in vitro* were investigated in **chapter 6**. Vaginal fibroblasts from prolapsed tissues produced matrices with different quality than the controls. Affected fibroblasts from postmenopausal prolapsed tissues produced stiffer matrices with high collagen content, and also fibres with less anisotropic orientation than those produced by controls.

These results were obtained from cells cultured on artificial substrates. Therefore I proposed a cell culture system using decellularized tissues to provide a more physiological microenvironment for the cells in order to study cell-matrix interactions in a disease-specific manner (**chapter 7**). This cell culture system was used to study the effect of prolapsed tissues in the phenotype transition to myofibroblast of fibroblasts derived from prolapsed and non-prolapsed tissues. In this pilot study we found that the stiffer muscularis layer of the decellularized matrices induced myofibroblast differentiation of POP, but not of control fibroblasts, and this differentiation was higher in the prolapsed matrices. Therefore, fibroblast to myofibroblast differentiation seems to be altered in cells from prolapsed tissues and also dependent on the extracellular matrix encountered.

Limitations

The studies presented in this thesis are a starting point for our understanding of the pathogenesis of pelvic organ prolapse of the bladder, and it is important for the reader to bear some limitations in mind.

The women recruited for these studies were from the same ethnic background, *i.e.* Caucasian Dutch women, and we know that ethnic origin is a strong predisposing factor for prolapse¹⁶. We chose the anterior compartment because it is the most problematic in the management of POP for Caucasian women, but care should be taken with expanding the results to other kinds of prolapse in a more general population. For the studies identifying the differences between acquired and intrinsic defects (**chapters 3 to 5**), very strict patient inclusion criteria were used. Despite the fact that the number of recruited women was based on a power analysis, the sample size is still too small to draw general conclusions. Furthermore tissues were taken after POP had occurred so it is difficult to say anything about the aetiology of the disease. The histological, biochemical and gene expression analyses were performed at a specific time point and therefore represent a “snap-shot” of the tissues at that moment; consequently we do not really know what happened before and after. As a result, it is difficult to draw conclusions about the cause and effect relationship of the differences identified. Nevertheless, thanks to the study design, it was possible to discriminate between intrinsic and acquired defects because each woman was her own control.

Several analyses were focused on collagen and collagen-remodelling factors because collagen is the most abundant protein in the anterior vaginal wall, and it plays a key role in the mechanical properties of soft tissues. Nevertheless, it is important to be aware that other proteins, particularly elastin, could also play an important role in prolapse and this should be investigated in more detail.

The cellular analyses were performed in *in vitro* set-ups, which allowed us to control certain parameters, but these set-ups do not fully reflect the *in vivo* situation. Moreover, we used only one cell type and *in vivo* there are other cells involved, *e.g.* macrophages, epithelial cells and smooth muscle cells. The use of *in vitro* models with co-culture systems could indeed provide even more relevant information about the pathogenesis of prolapse¹⁷. In **chapter 6** we had a small sample size and **chapters 2 and 7** were only small pilots.

Regardless of these limitations, results from our cellular studies are of great importance as we show that prolapsed tissues alter fibroblasts functionalities, matrix production and cell-matrix interactions *in vitro* in the presence of different surface substrates, including native tissues and stiff tissue-culture plates. Since the polymeric meshes currently used in genital prolapse surgery are stiffer than the native tissues in humans¹⁸⁻²⁰, new questions arise, such as: how does the introduction of stiff polymeric meshes affect the microenvironment of the already altered prolapsed tissues? How would

that affect cell-matrix interactions? Would mechanical loading ameliorate or worsen the situation? If so, how? And how can we use this information in the clinical practice?

Model of “frustrated healing”

Taken together, the results from this thesis provide evidence that supports the hypothesis that changes in cell behaviour (**chapters 2, 3, 5 and 6**) and tissue composition (**chapter 4**) in the majority of Dutch Caucasian women with pelvic organ prolapse of the bladder are acquired and not intrinsic. Moreover, it was established that surface substrate affects cellular behaviour, and that cell–matrix interactions seem to be impaired in fibroblasts from prolapsed tissues (**chapter 7**). The changes seen here in the women with POP seem to be a consequence of the prolonged exposure to the prolapsed weakened tissues.

But how did the tissues become weak to begin with?

The pelvic floor supportive tissues are constantly being loaded by changes in the intra-abdominal pressure. Soft tissues that are constantly loaded are also constantly being remodelled. To keep the tissues strong, cells need to feel a constant basal loading to maintain homeostasis between tissue degradation and production. When the required basal loading is disturbed by excessive mechanical loadings, such homeostasis can be lost, leading to acute or chronic soft-tissue damage.

It is well known that the most common risk factors of POP are related to excessive mechanical loading, *e.g.* pregnancy, parturition, obesity, chronic coughing and heavy lifting¹⁶. If the pelvic floor supportive soft tissues are not properly healed after an injury, *i.e.* when the gap created by the wound is too big, or if the loads applied to the tissues are too great, then the constant loading of the pelvic floor could lead to “frustrated wound healing”, and consequently to weak tissues. This could create a negative feed-back loop where cells trying to compensate for the lack of tissue strength could activate at least two molecular pathways (identified in **chapter 4**). One pathway is related to ECM production and integrins, which act as the mechanosensors of the cells and help transmitting loads. The other pathway is related to muscle cells and cellular contraction and should have direct effects on wound healing, particularly in myofibroblasts as they share characteristics of muscle cells and are in charge of closing wounds during tissue repair. Either way, the end result is that cells become unable to restore the extracellular matrix in the correct way, resulting in permanent changes to tissue composition, tissue mechanical properties and cell behaviour, leading to eventual tissue prolapse (Figure 1). Whether or not these changes in tissue composition and cell behaviour are reversible, and indeed how, still needs to be explored.

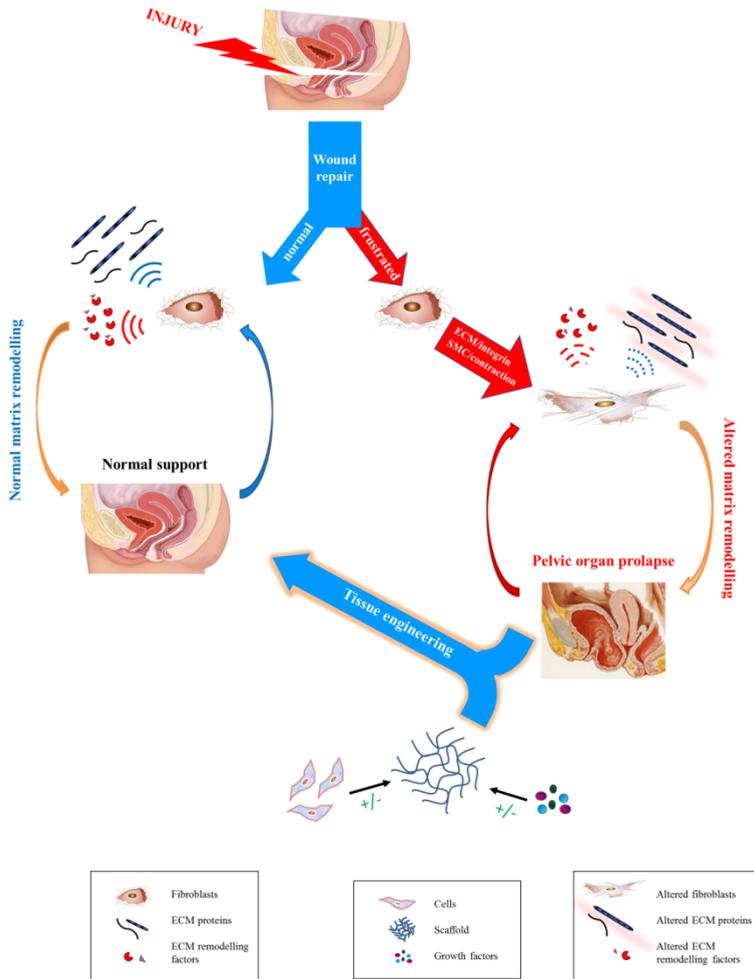


Figure 1. Model of development of pelvic organ prolapse after “frustrated healing” of an injury and how we could re-establish support with a tissue engineered implant. Under normal conditions, when an injury occurs in the pelvic floor supportive tissues and normal wound healing repair process takes place, normal fibroblasts help to restore support by producing extracellular matrix (ECM) proteins and remodelling factors (blue arrow, left panel). If the pelvic floor supportive soft tissues are not properly healed after an injury (red arrow, right panel), then the constant loading of the pelvic floor could lead to “frustrated wound healing”, and consequently to weak tissues. This could create a negative feed-back loop where cells trying to compensate for the lack of tissue strength could activate at least two molecular pathways: (i) ECM production and integrins; (ii) smooth muscle cells and cellular contraction. Cells become unable to restore the ECM in the correct way, resulting in permanent changes to tissue composition, tissue mechanical properties and cell behaviour, with eventual tissue prolapse. In these women with acquired defects due to prolapse, support could be re-established by using tissue engineering implants with a scaffold with or without cells and/or growth factors to promote tissue regeneration.

Implications for patient care

The new knowledge acquired from the studies presented in this thesis has implications for clinical management of POP as well as for the development of new treatments. The use of the model of “frustrated healing” presented here as a hypothetical cause of prolapse for a large female population, should lead to the opening of new lines of research into understanding these processes, and understanding how and when they are triggered. To that end it would be imperative to identify and quantify different kind of loadings: those required to keep tissue homeostasis, those that could lead to tissue damage, and those that could promote tissue regeneration.

The application of this knowledge would change patient care, as we would be able to advise women with regard to “recovery times” after an injury has occurred, *e.g.* after delivery, also about certain movements they should avoid, and even to tailor exercises to best strengthen the pelvic floor. Moreover, if obstetricians are able to identify if the gap created by a wound after an injury is sufficiently big that a “frustrated healing” would be likely to occur, then a decision can be made as to whether or not this patient would require provisional support to facilitate the healing process. Some women would still be predisposed to prolapse, ethnically and/or genetically. Development of new diagnostic tools to properly identify those women with a predisposition to POP will allow us to develop personalized preventive strategies to further improve primary care.

Preventive strategies might take several years to be applied in clinics, meanwhile it is expected that the ageing female population will double by 2030, therefore the prevalence of pelvic organ prolapse will increase²¹. When women develop POP late in life they might require treatments that provide permanent anatomical support as the capacity of tissue regeneration decreases with ageing. The meshes now available in the market provide good anatomical support, but irreversible consequences such as erosion and extrusion are unacceptable and development of new implants is an urgent challenge.

Why are the available surgical treatments not optimal?

The currently available surgical treatments for pelvic organ prolapse of the anterior compartment aim to restore anatomical support. Native tissue repair is considered the golden standard. Unfortunately this has high recurrence rates²² that could be partly explained by the acquired defects observed in women with POP. Since the tissue has changed and the fibroblastic cells seem to be unable to remodel their ECM in a proper way it is not surprising that the tissues remain weak and fail again after the operation. A more permanent treatment is the use of polypropylene braided meshes to restore anatomical support. Unfortunately, the prevalence of irreversible complications have made the use of these meshes very controversial and there is still not enough evidence to support their use to repair the anterior compartment²³.

Why are these meshes failing?

These meshes were not originally designed for pelvic floor repair, but rather to repair abdominal hernias. The vaginal mucosa is a very different microenvironment than the abdominal skin, and the wound repair process might be different as well. As evidence of the adverse effects of the meshes grows, so does our understanding of the implant-tissue interactions. The polypropylene braided meshes were designed to be macro-porous in an attempt to promote tissue integration. The formation of fibrotic tissues around the meshes is seen as a successful integration, but if granulose tissues are not properly remodelled, the encapsulated meshes can be detrimental to the success of the implant because the tissue-implant complex will have different composition, architecture, and mechanical properties than the rest of the soft tissue of the anterior vaginal wall. This mismatch between the different layers of the tissue-implant complex leads to shear stresses at the implant surface, the development of myofibroblasts and subsequently meshes contraction, one of the most common complications of the implants.

Polypropylene meshes are stiffer than the native tissues¹⁸⁻²⁰. The mismatch between the mechanical properties of the meshes and the surrounding tissues could result in what is known in the orthopaedic field as “stress-shielding”. When this occurs, the load that is normally transmitted to the tissues will solely be borne by the implant (the component with the highest elastic moduli), causing a degenerative tissue remodelling and eventually implant failure. Poor implant-tissue integration can be further affected by infections, inflammatory reactions and the poor matrix remodelling capacity of the cells in the host-tissues. Thus there are many factors that can contribute to the failure of implants and I found it particularly surprising that the meshes work for 80%²⁴ of the women treated, as they provide good anatomical support but do not necessarily integrate well into the host-tissues. Still, considering the large number of women involved, a 9.6%²⁴ failure rate is unacceptable.

Evidence of the adverse effects of the meshes is increasing and it is clear that a new generation of implants is required. These new implants should be specifically designed for the constantly loaded environment of the vaginal wall of patients that need surgical treatment for pelvic organ prolapse. Therefore, the new implants should be designed with mechanical properties that match those of the host-tissues to avoid stress-shielding, but at the same time are stiff and strong enough to provide proper anatomical support. Moreover, results from this thesis point towards the need to develop new treatments that not only restore the anatomical function but also optimize the local environment of the pelvic floor to re-establish the proper functioning of the supportive tissues and the cells involved.

Tissue engineering strategies

The use of autologous cell-based tissue engineering implants for pelvic floor repair could be an interesting choice for women that have acquired defects as a consequence of prolapse without having a genetic predisposition (Figure 1). Tissue engineered implants require a choice of scaffolding material and living cells grown together, typically *in vitro*, before being implanted into the patient. This raises other issues such as the choice of cells (stem cells, iPS, fibroblasts, etc.), the material for the scaffold and whether or not growth factors are required to promote tissue regeneration. Since surface substrate influences cell behaviour and cell-matrix interactions seem to be altered with POP, good implant integration can be promoted by modulating the surface substrate and the conditions under which these interactions occur. Identifying the “right” components for the ideal implant is definitely a challenge that should be undertaken in order to improve clinical management of pelvic organ prolapse.

How to move forward?

The use of *in vitro* models to study cell-matrix interactions can be very valuable to increase our understanding of the pathophysiology of prolapse and thereby to improve treatments. Our results, and those from others, suggest that fibroblast to myofibroblast differentiation is altered in cells from prolapsed tissues, and that this process depends on the extracellular matrix encountered by the cells. Myofibroblasts are involved in tissue repair and regeneration²⁵, and identifying different pathways that lead to myofibroblast differentiation in fibroblasts from prolapsed tissues could provide interesting insights into the progression of prolapse. The acquired defects observed in cell behaviour, even after the cells have been extracted from their natural microenvironment, seemed to be permanent. Studies into the epigenetics of these changes could give clues about the causes and how to reverse them if possible. Besides, since fibroblasts’ behaviour depends on the surrounding matrix, and POP and control cells behave in different ways when exposed to prolapsed matrices, the effect of matrix components and growth factors is worth exploring in a disease-specific manner. It would also be of great relevance to identify which matrix components dictate the phenotype of the fibroblasts and the dynamics of this process under normal and pathological conditions.

Since cell-matrix interactions are greatly influenced by mechanical stimuli and the pelvic floor is constantly being loaded, future studies should include dynamic *in vitro* models to simulate physiological and pathological microenvironments. To that end it is imperative to identify the range of loading that leads to tissue damage, and the loading range that promotes tissue regeneration. Information from such studies can be used to identify optimal microenvironments that can be used to design the “ideal implant”. Once the implant is made, *in vitro* models can also serve as part of a first screening of possible

therapies for prolapse, before using animal models and prior to clinical implementation. These screenings can be designed to be personalized as cells from the person requiring treatment can be tested *in vitro* to determine the suitability of specific treatments for her.

The use of animal models that properly resemble the female human pelvic floor environment is difficult, due to the anatomical, morphological and biomechanical differences between species. Currently there is no animal model of prolapse, so it is difficult to interpret results from “non-prolapsed tissues”. Creating such a model would increase the probability of success of the new implants as development of new therapies for prolapse should be tested in diseased models, specifically because of the changes to the fibroblasts and their microenvironments. After the safety and effectiveness of new devices have been established *in vitro* and *in vivo* (animal models), then we can think about using the implants in proper clinical trials before using them in the treatment of patients.

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