Autologous plasma coating - a new approach for improvement of the biocompatibility of mesh implants

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LIST OF FULL PAPERS RELATED TO THE SUBJECT OF THE THESIS

I **H. Gerullis**, E. Georgas, C. Eimer, P. Goretzki, B. Lammers, B. Klosterhalfen, M. Boros, M. Wishahi, G. Heusch, T. Otto (2011) Evaluation of biocompatibility of alloplastic materials: development of a tissue culture *in vitro* test system. *Surg Technol Int. 2011 Dec 1;XXI:21-27* *IF: 0*

II **H. Gerullis**, B. Klosterhalfen, M. Boros, B. Lammers, C. Eimer, E. Georgas, T. Otto. IDEAL in meshes for prolapse, urinary incontinence and hernia repair. *Surg Innov. 2013;20(5):502-8* ***IF: 1.537***

III **H. Gerullis,** Georgas E, Eimer C, Arndt C, Barski D, Lammers B, Klosterhalfen B, Boros M, Otto T. Coating with autologous plasma improves biocompatibility of mesh grafts in vitro: development stage of a surgical innovation. *Biomed Res Int.* 2013;2013:536814. doi: 10.1155/2013/536814 ***IF: 2.88***

IV **H. Gerullis,** Georgas E, Boros M, Klosterhalfen B, Eimer C, Arndt C, Otto S, Barski D, Ysebaert D, Ramon A, Otto T. Inflammatory reaction as determinant of foreign body reaction is an early and susceptible event after mesh implantation. *Biomed Res Int.* 2014;2014:510807. doi: 10.1155/2014/510807 ***IF: 2.88***

V D. Barski, T. Otto, **H. Gerullis**. Systematic Review and Classification of complications after anterior, posterior, apical, and total vaginal mesh implantation for prolapse repair. *Surg Technol Int. 2014 Mar;24:217-24* *IF: 0*

LIST OF SELECTED ABSTRACTS RELATED TO THE SUBJECT OF THE THESIS

1 **H. Gerullis,** C. Eimer, E. Georgas, B. L. Ammers, P. Goretzki, B. Klosterhalfen, M. Boros, A. Ramon, T. Otto. Improved biocompatibility of meshes used for hernia, incontinence and organ prolapse repair by plasma coating-results of *in vitro* and *in vivo* studies. *Eur Urol Suppl 11(1) pp. E1068-U229 (2012)*

2 **H. Gerullis**, E. Georgas, C. Eimer, B. Lammers, P. Goretzki, B. Klosterhalfen, M. Boros, A. Ramon, T. Otto. Evaluation of biocompatibility of alloplastic materials - development of a tissue culture *in vitro* test system. *Eur Urol Suppl, 11(1), 2012, e807a*

3 **H. Gerullis**, C. Eimer, J. Bagner, T. Otto. *In vitro* test pattern for determination of biocompability of alloplastic materials used for treatment of urinary incontinence. *Urology, Volume 74, Issue 4, Suppl, October 2009, S232*

LIST OF ABBREVIATIONS

FBR	foreign body reaction
FDA	Food and Drug Administration
POP	pelvic organ prolapse
SUI	stress urinary incontinence
PVDF	polyvinylidene fluoride
CD	cluster of differentiation
PBMCs	peripheral blood mononuclear cells
ATR	Advanced Tissue Regeneration System
TVT	Tension-free Vaginal Tape
DAAD	Deutscher Akademischer Austauschdienst (German Academic Exchange Service)
DFG	Deutsche Forschungsgemeinschaft (German Alliance of Science Organizations)
PTFE	polytetrafluorethyle
MMP-2	matrix metalloproteinases-2
IDEAL	Innovation, Development Exploration, Assessment and Long-term
PP	polypropylene
PET	polyethylene terephthalate

1. SUMMARY

Two recent warnings by the US Food and Drug Administration (FDA) relating to severe side-effects led to discussions concerning the biocompatibility requirements of surgical meshes. There are currently no standardized tools for the comparison of surgical meshes.

Our aim was to develop a standardized and manufacturer-independent *in vitro* test system for the adherence performance of tissue clusters (fibroblasts, endothelial cells and muscle-derived cells) as a marker for the biocompatibility of commercially available meshes. In this test system, we could establish a repeatable ranking of meshes with regard to their biocompatibility. The adherence behavior was independent of the individual patient features, suggesting that the biological behavior of a mesh is probably conditioned by the structure of the biomaterial or/and its chemical composition rather than by individual host characteristics/features. This *in vitro* test system has been shown to be a feasible pattern for the investigation of different mesh coating strategies. The coating of meshes prior to cultivation, *e.g.* with peripheral blood mononuclear cells (PBMCs), did not affect the adherence score, whereas coating with platelets and blood plasma increased the score, suggesting improved biocompatibility *in vitro*. Plasma coating exhibited the greatest potential to improve the *in vitro* adherence score. The previous ranking of native meshes remained consistent after coating, but established at a higher level.

In order to explore the predictive value and validity of the test system and also newly tested coating strategies, we translated the preliminary *in vitro* results into *in vivo* circumstances and conducted a large-animal experiment on sheep. The entire experimental approach followed the recently developed recommendations of the IDEAL (Innovation, Development Exploration, Assessment and Long-term) study for surgical innovations. In this long-term animal study, we demonstrated that our recently developed *in vitro* test system may predict the *in vivo* performances of the meshes. This effect was independent of the location of the mesh in the body, although its particular extent varies with the site of implantation. The coating of meshes with autologous plasma prior to implantation had positive effects on the biocompatibility of meshes *in vivo*. Investigation of the ultra-short-term determinants of the foreign body reaction (FBR) at the implant site *in vivo* revealed that the local inflammatory reaction is an early and susceptible event after mesh implantation. It cannot be influenced by prior plasma coating and does not depend on the localization of implantation. The project is continuing, the method currently being implemented in humans.

2. INTRODUCTION

2.1. Biocompatibility and surgical meshes

The mechanical and biocompatibility properties of synthetic materials used to replace or support native tissues play a crucial role in their *in vivo* function. An ideal graft material is expected to be chemically inert, non-toxic, non-allergic, non-inflammatory, resistant to infection, non-carcinogenic, solid, sterilizable and convenient and affordable.[2]

Biocompatibility is defined as "the ability of a material to perform with an appropriate host response in a specific situation", implying a symbiotic relationship of acceptance between the host and the respective implanted material.[3] For a material to perform best, it needs to be integrated properly into the tissue, to generate an appropriate inflammatory response and to maintain mechanical integrity (keep its shape). These qualities are discussed along with additional and host-related factors that contribute to biocompatibility. As biocompatibility is described by the FBR at the host-tissue/biomaterial interface, three crucial steps have been identified that describe the time course of the FBR: protein absorption, cell recruitment and, finally, fibrotic encapsulation and extracellular matrix formation. The dynamics of the FBR is greatly influenced by the biomaterial composition, and in particular the type of polymer, the material weight, the filament structure and the pore size. In this regard, *Amid* proposed a standardized classification system to distinguish between different types of synthetic meshes, in particular emphasizing the physical properties of biomaterials and their predictable impact on possible adverse events (**Table 1**).[4] They classified meshes into 4 different groups, focussing mainly on the respective pore size. They assumed that the utilization of meshes from the different groups has a prognostic value for different adverse events, such as the risk of infection, seroma formation and fistula formation, and may therefore be used as predictors for biocompatibility. They recommended that the classification should influence the clinical decision as to which alloplastic material to apply in a particular clinical situation. In accordance with those early results, it is currently accepted that large porous meshes lead to the best tissue integration and, thanks to the reduced surface area, produce the least FBR, inflammation and fibrosis. Small-pore or microporous and foil- or layer-like mesh modifications, however, are associated with a significantly greater FBR and inflammation frequently related with the phenomenon of bridging, which finally may cause a significant contraction or shrinkage of the mesh. Polypropylene (PP), polyethylene terephthalate (PET) and polytetrafluoroethylene (PTFE) are currently the most commonly used mesh materials, whereas PP seems to be the most frequently used polymer for the construction of surgical meshes.[5] There appears to be a trend in current mesh development

toward minimization of the mesh density and the use of macroporous weave patterns of monofilament PP.[6] In a review analyzing the mechanical properties of and the tolerance to synthetic implants for stress urinary incontinence (SUI) and pelvic organ prolapsed (POP), *Cosson et al.* identified PP (known to offer durability and elasticity) as the most promising material for those indications.[7] Biocompatibility assessment of alloplastic materials through the use of appropriate cell cultures *in vitro* is a valid and accepted method which furnishes information about the toxicity of the investigated material, and the possible effects on the metabolism and growth of the cells.[8] Although cells can be sensitively characterized with this method, and the conditions can easily be standardized, no complex tissue representative of the human body has yet been investigated.

Table 1. The *Amid* classification

	Characteristics	**Examples**
1	A completely macroporous mesh, >75 µm	Prolene Marlex Atrium Trelex
2	A completely microporous mesh, <10 µm	Gore-Tex surgical membranes
3	A macroporous patch with multifilaments or a microporous component	Mersilene (woven Dacron) Teflon (PTFE) Surgipro (woven PP) MycroMesh (perforated PTFE)
4	Submicronic pores, often associated with type 1 materials to prevent adhesion (*e.g.* peritoneum)	Silastic Celigard Dura mater substitute

2.2. Foreign body reaction

When implanted for a particular indication, a mesh represents a foreign body which induces a FBR, which is an important surrogate for its biocompatibility performance. This reaction is triggered by the initial acute phase reaction and the subsequent construction of the implant matrix, mostly conducted by the migration of fibroblasts producing glycosaminoglycans and collagen. The FBR has been histologically described as a foreign body granuloma adjacent to the mesh fiber and a surrounding collagen capsule that shields

the host from the foreign material. It seems likely that such a chronic inflammatory process impairs normal wound healing and tissue regeneration and may result in reduced functionality and increased side-effects when applied clinically.[9] A considerable influence on the dynamics of the FBR is exerted by the biomaterial composition, and in particular the type of polymer, the material weight, the filament structure and the pore size.

However, the process of FBR does not necessarily reduce the proposed mesh function of restoring mechanical functionality in a particular region of the body. Several attempts have been made to improve the biocompatibility of meshes and reduce the FBR.[10, 11] The exact FBR mechanisms and respective time flow *in vivo* are not entirely understood, but the rapid accumulation of huge numbers of phagocytic cells, and especially blood monocytes and tissue-derived macrophages, play a crucial role in it.[12-14] Additionally, the transcriptionally-induced overexpression of the matrix metalloproteinases-2 (MMP-2) seems to play a key role in the FBR.[15-18] New therapeutic strategies may target cellular and molecular interactions during these phases, influencing the complex cascade of immune modulators, soluble mediators and different cell types. A beneficial effect of gentamycin on a chronic FBR by modulation of MMP-2 gene transcription has recently been described, which may be a feasible approach to the optimization of mesh integration into the abdominal wall, and ultimately to improvement of the long-term outcome following hernia mesh repair.[19]

2.3. Application of surgical meshes and current controversies

Alloplastic materials such as meshes are widely applied in surgical approaches for hernia, incontinence and prolapse situations. The development of meshes is an ongoing process characterized by changes in polymer structure, biocompatibility, operative handling and costs. A good mesh should provoke a negligible FBR with no pathologic fibrosis at a decreased risk of infection. In a Public Health Notification issued in 2008, the FDA reported more than 1000 unexpected and severe adverse events, mostly associated with transvaginal placement of a surgical mesh to treat POP and SUI.[20] In 2011, a second FDA warning was announced on the basis of 2,874 newly identified Medical Device Reports: 1,503 associated with POP repairs, and 1,371 associated with SUI repairs.[21] Factors involved included the overall health of the patient, the surgical technique used and concomitant procedures undertaken; particular importance was attached to the mesh material and the size and shape of the mesh as causes of adverse events. However, the search for the optimal mesh for a particular indication, with high functionality and biocompatibility and a minimized side-effect profile in the short-term and long-term follow-up remains difficult. These two FDA

warnings have led to several regulatory changes for surgical meshes, including the upgrading of risk classifications, requirements for clinical studies to address the risks and benefits of meshes used to treat POP and SUI and the expanded post-market monitoring of device performance.[21]

2.4. The IDEAL recommendations

In the assessment of quality standards for surgical meshes, comparability with other meshes with regard to quality and stage of development should be possible. Despite the existence of several models for the assessment of different meshes with regard to their particular biomechanical characteristics, there are currently virtually no standardized tools for comparisons among meshes.[22, 23] Many alloplastic materials are still being used without proper trials and are recommended by manufacturers rather than on the basis of data arising from *in vitro* or *in vivo* experiences. Once a product is on the market, financial support for further investigations decreases and ongoing evaluation with unknown results is often not desired. Thus, as compared with the strict regulations for drug development and market implementation, the process of adopting and improving surgical innovations (*e.g*. meshes) is still unregulated, unstructured and variable. In 2009, the Lancet dedicated a series to the topic of "Surgical Innovation and Evaluation" and its current status.[24-26] A five-stage description of the surgical development process has been proposed, the IDEAL model which allows the assignment of every surgical innovation, *e.g.* surgical technique, alloplastic materials, *etc.*, to its particular corresponding step of development. The IDEAL framework, has so far been used in retrospective studies, and in particular in the description of surgical procedures.[27, 28] However, this framework is highly recommended for the application in the development of surgical innovations different from surgeries. This is important as the innovations are often introduced and developed only by manufacturers. Research ethics committees and device regulatory bodies could help by requiring a declaration of the IDEAL stage that the investigators/manufacturers feel the device or procedure has reached, with supporting evidence. Innovations in the idea stage, for instance, would then be expected to lead to proposals for a prospective development study. Thus, application of IDEAL would allow the setting of minimum requirements for developmental steps prior to market entry for any innovative product or device. As the current studies cover the topic of a surgical innovation, we followed the IDEAL recommendations in the entire investigation strategy and course in order to ensure comparability, visibility and confirmability.

3. MAIN GOALS

There are currently no standardized tools for the comparison of surgical meshes. The initial purpose of our studies was to develop and standardize an *in vitro* test system with which to investigate biocompatibility features of surgical meshes. Once having developed this test system, we aimed to implement mesh-coating strategies and subsequently to investigate their influence on biocompatibility in *in vitro* performance. In order to explore the predictive value and validity of the test system and also newly tested coating strategies, we conducted a large-animal experiment in sheep. The entire approach followed the recently developed recommendations of IDEAL for surgical innovations.

1. Study I. The main purpose was to investigate and develop an *in vitro* approach for an assessment of the biocompatibility features of surgical meshes. Seven different mesh types, currently used in various indications, were randomly selected and microscopically investigated after incubation for 6 weeks with regard to their adherence performance, using a tissue culture approach, with tissues representative of fibroblasts, muscle cells and endothelial cells, originating from 10 different patients.

2. Study II. We aimed to investigate different mesh coating modalities with autologous blood components and their impact on the biocompatibility performance of the meshes *in vitro*, using the previously developed test system. Seven different mesh types were therefore coated prior to cultivation with autologous PBMCs, platelets and blood plasma and subsequently incubated for 6 weeks in a minced tissue assay. The adherence performance of the tissues on the meshes was investigated microscopically, assessed semi-quantitatively and compared with the native counterparts, using a previously developed scoring system.

3. The goals of ***Study III*** and ***Study IV*** were to translate the preliminary *in vitro* results into an animal model in order to validate the *in vitro* test system and to explore its predictive value for *in vivo* surroundings. Three different meshes [TVT (Tension-free Vaginal Tape), UltraPro® and polyvinylidene fluoride PVDF] with different previous *in vitro* performance scores were implanted in female sheep in a native or a plasma-coated version. In the ultra-short-term study, meshes were explanted and investigated histochemically for inflammatory infiltrate, macrophage infiltration, vessel formation, myofibroblast invasion and connective tissue accumulation at the implant site at 5 min, 20 min, 60 min and 120 min. In the long-term study, meshes were explanted after 3, 6, 12 or 24 months and processed.

4. MATERIAL AND METHODS

4.1. In vitro experiments

4.1.1. Meshes/Patients

We randomly identified alloplastic materials currently applied as implants for different surgical indications covering hernia repair, POP and SUI. A total of 7 different meshes were investigated in this study. The meshes and their biomechanical/material characteristics are listed in **Table 2**. The alloplastic materials were prepared in 2 x 2 cm fragments for further investigations. Additionally, we harvested tissue probes of muscle, fascia and renal vein from 10 patients undergoing right-side nephrectomy. All patients gave their informed consent previously. The tissues and cells were processed identically in all patients. Each mesh was tested with the tissue and cells of each patient for comparison purposes.

Table 2. Investigated meshes and their characteristics

Mesh name	Material	Biomechanical characteristics
Vitamesh®, ProxyBiomedical	large-pore monofilament polypropylene	knitted PP, pore size 2410 µm, thickness 250 µm, tear resistance (F_{max} N) 33.7
Dynamesh®, FEG Textiltechnik	monofilament PVDF	effective porosity: 58%, reactive surface: 1.97 m²/m², suture pull-out strength: 31 N, tear propagation resistance: 28 N, pore size: 3000 µm
TFT Motifmesh®, ProxyBiomedical	micromachined PTFE	pore size 235 µm, thickness 150 µm, tear resistance (F_{max} N) 15.1
TVT PP	PP	non-absorbable, permanent PP suture, pore size 164 x 96 µm
UltraPro®, Hernia System Medium UHSM®, Ethicon	PP reinforced with polyglecaprone fibers	filament thickness 0.09 mm, mesh thickness 0.5 mm, F_{max} N 69 N, pore size 300 µm
Proceed surgical mesh®, Ethicon	monofilament PP encapsulated with polydioxanone (PDS)	closely knitted with small pores < 1000 µm size, high tensile strength
Mersilene, Johnson & Johnson	multifilament mesh, PET	density 0.19 g/cm^3, pore size 120-85 µm

4.1.2. Tissue preparation and mesh incubation

In an initial cell culture approach involving investigation of the cells of the different tissues, we could not identify adherence on the mesh microscopically. In contrast, the cells grew on the bottom of the cell culture device. When performing a tissue culture, we observed tissue adherence both on the meshes and on the bottom of the cell culture device. As the cells did not grow on the respective mesh matrices, we decided to use tissue cultures for the following investigations. In addition, we considered tissue cultures more appropriate because of the reduced artificial modification processes due to the shorter culture processing.

We extracted tissue probes originating from muscle, fascia and renal vein at a length of 0.5-1.0 cm each from 10 different patients. After crushing, we incubated the tissue with phosphate-buffered saline and, after 2 additional washing procedures, incubation was performed with DMEM/F12 plus 10% serum and 1% glutamine + 1% penicillin/stretomycin. After successful expansion and growth (80-90% adherent growth) of the tissue pellets, the different alloplastic materials were added. Thus, the prepared and expanded tissue probes consisting of myoblast, endothelial cells and fibroblasts presenting relevant tissues of the pelvic floor were used *in vitro* in order to create a model for investigation of the integrity of the different mesh types. Myoblasts were detected by α-sarcomeric actin and desmin as markers of myogene differentiation. Fibroblasts were stained with antibodies targeting vimentin, whereas antibodies against cluster of differentiation 34 (CD34) were used for the verification of endothelial cells. We reproduced every single approach 10 times with tissue probes from the 10 different patients.

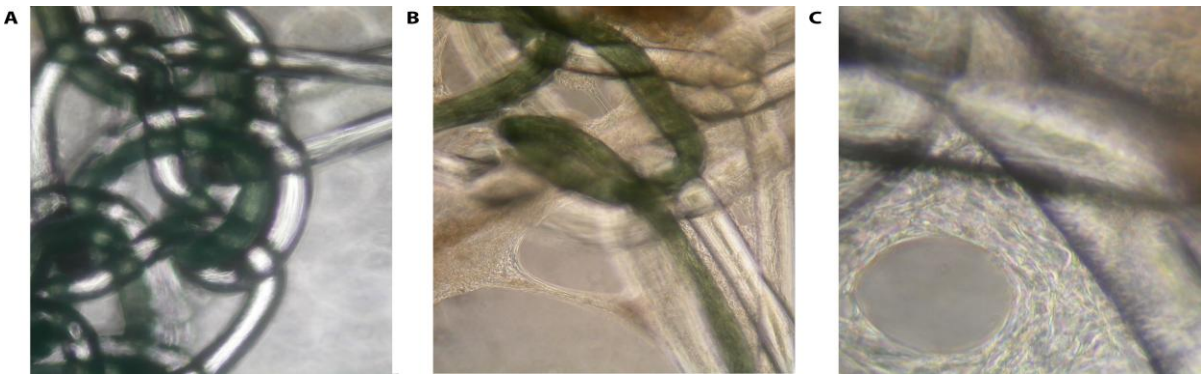


Figure 1. *Microscopic adhesion course on PVDF:* ***A)*** *native prior to tissue culture,* ***B)*** *after a 3-week tissue culture and* ***C)*** *after a 6-week tissue culture (time point of assessment). In* ***B)*** *and* ***C)*** *the spaces between the mesh filaments are increasingly occupied by fibroblasts, collagen deposits and capillaries. As adherence appeared 3-dimensionally, entirely defined pictures cannot be shown.*[29]

4.1.3. Mesh coating

PBMCs, platelets and plasma were used. PBMCs were separated through density gradient centrifugation using Ficoll.[30] For the isolation of platelets and the respective mediators, the Advanced Tissue Regeneration System (ATR® by Curasan Inc) was used (*http://www.curasan.de/de/produkte/dental/atr/atr.php*). The plasma preparation procedure followed the classical method of *Crowley*.[31] After isolation of the 3 different blood components of each patient, we incubated the meshes (2 x 2 cm) with 10 ml of the respective suspension and incubated them for 12 h prior to testing with tissue. Successful plasma coating is exemplified in **Figure 2**.

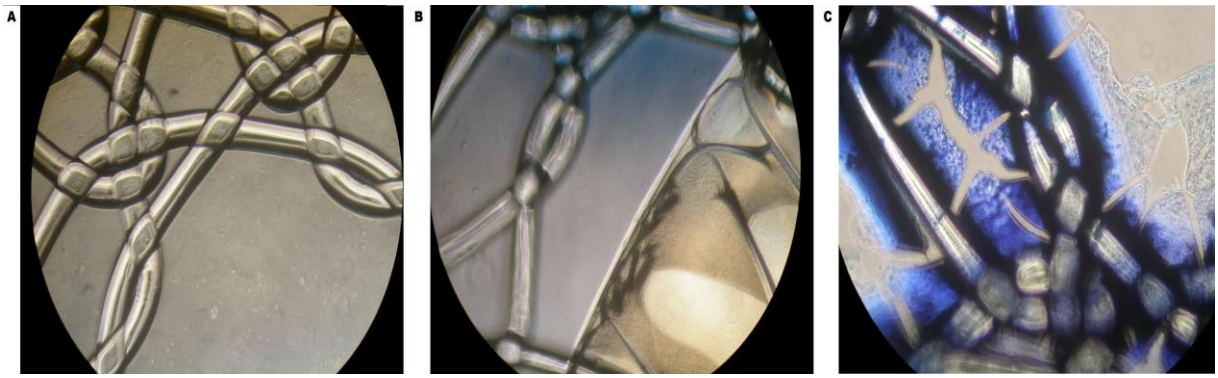


Figure 2. *PVDF: **A**) native, **B**) after a 12-h plasma incubation, **C**) after a 12-h plasma incubation and trypan blue staining. In **B** and **C** the plasma is adherent to the mesh filaments, whereas the non-covered parts of the mesh appear native as in **A**.*[32]

4.1.4. Morphological study

The adherence and the cell count (if possible) were assessed microscopically and through the use of immunohistochemistry after co-incubation of the cells with different types of alloplastic meshes. The test duration was 6 weeks. Meshes were investigated with regard to interstructural tissue connections and the quantity of mesh-adherent cells. Tissue cultures were maintained up to 4 months, with frequent changes of the medium, and assessment was repeated if possible.

A descriptive/semiquantitive assessment pattern was used in order to describe the adherence of tissue to the investigated mesh materials. The assessment pattern was based on the maximum identifiable quantity of mesh-adherent cells within a tissue cluster per vision field. Adherence performance was ranked after assessment of the quality and quantity of the tissue clusters/cells as none, fair, good or excellent.[33]

4.2. In vivo experiments

4.2.1. Animals

The animal experiments were conducted at the Institute for Surgical Research of the University of Szeged, Hungary, in accordance with the NIH Guidelines (Guide for the Care and Use of Laboratory Animals). The experimental protocol was approved by the Animal Welfare Committee at the University of Szeged (license/permission No. V01353/2010).

Fourteen 6-month old, female sheep weighing 20-25 kg were housed and cared for at the University farm for experimental animal studies. We included 2 animals more than the 12 needed for safety calculations. All the animals had free access to food and water, and were cared for by an educated keeper and routinely inspected by a veterinarian. On the basis of the previously described test system and the resulting ranking, we selected 3 meshes representing good, intermediate and poor *in vitro* performance; PVDF, UltraPro® and TVT (**Table 3a**).

4.2.2. Mesh implantation

Operations were performed on sheep in a supine position. The animals were intubated and an aspiration tube was introduced into the stomach. Anesthesia with Isoflurane 2% mixed with air and O_2 (50/50%) was then established. Surgery was performed by using a longitudinal laparotomy. We chose 3 different locations in the sheep to implant the meshes via open surgery. In order to represent different *in vivo* surroundings, 3 meshes were placed in the following localizations: a) interaperitoneally, b) as fascia onlay and c) as muscle onlay (fascia sublay). The size of the implanted meshes was 3 x 5 cm. Then, 3 plasma-coated versions of the same mesh type were implanted in equivalent localizations on the contralateral side of the torso. Meshes had to be incubated with autologous plasma at least 12 h prior to implantation. This procedure was performed in 14 animals, resulting in 4 animals per mesh type (plus 2 animals with PP TVT/PVDF). The mean operation duration was 1.5 h.

For the short-term study, 3 additional female sheep weighing from 20 - 25 kg and at least 6 months old were included. The protocol for housing and veterinary maintenance was as in the long-term study. The size of the implanted meshes was 3 x 12 cm. For every native mesh implant, a respective plasma-coated version was implanted in an equivalent localization on the contralateral side of the torso. The length of incubation prior to surgery was at least 12 h. We selected 1 sheep per investigated mesh, *i.e.* resulting in 3 animals. The investigated meshes were again PVDF, UltraPro® and TVT. The chosen time points for explantation were 5 min, 20 min, 60 min and 120 min. At each explantation time point, we dissected a piece about 3 x 3 cm in size from the initially implanted mesh. During implantation, the meshes

were fixed with 2 sutures at both ends. The mean operation duration for the implantation was 50 min.

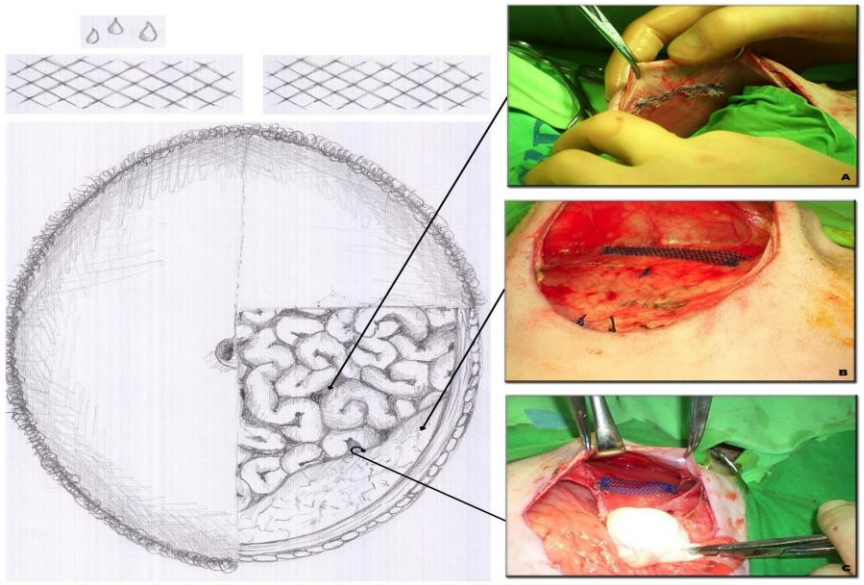


Figure 3. *Intraoperative sites during implantation: (**A**) intraperitoneal, (**B**) fascia onlay, (**C**) muscle onlay. 12 h prior to implantation the meshes were coated and incubated with autologous plasma. Meshes were implanted bilaterally into the torso to allow intraindividual comparison of coated versus uncoated meshes per animal.*

4.2.3. Mesh explantation

After 3, 6, 12 and 24 months, three animals, respectively, underwent surgery for mesh explantation. The meshes were identified and then harvested, extent of local reactions was described macroscopically. The animals were sacrificed directly after mesh explantation and harvesting of probes of parenchymatous organs (liver, intestine, kidney, lung, heart). The harvested material was then assessed for foreign body reaction, scar formation and inflammatory reaction. For the short term study, explantation time points were 5 min, 20 min, 60 min, 120 min, respectively.

4.2.4. Morphological studies

A single longitudinal section of mesh and adhesive tissue was obtained from each explanted mesh. Tissue samples were fixed in 10% formalin, and then sliced into 0.3 x 1 cm pieces and embedded in paraffin. In each case, 10 to 15 sections of 4 µm thickness were stained with hematoxylin and eosin (H&E), and with periodic acid-Schiff plus diastase and Elastica van Gieson. All mesh specimens were studied by light microscopy which was controlled by immunohistochemistry performed on the material embedded in paraffin, using

the avidin-biotin complex method with diaminobenzidine as a chromogen. The procedure was repeated twice for every sample. The antibodies used in this study included polyclonal rabbit anti-human CD3, 1:50, as pan-marker for T-lymphocytes (DAKO, Hamburg, Germany), polyclonal rabbit anti-human CD138, 1:50 as pan-marker for plasma cells (DAKO, Hamburg, Germany), monoclonal mouse anti-porcine CD68, 1:50 (DAKO, Hamburg, Germany), as pan-marker for macrophages, monoclonal anti-human CD15, 1:10 (Becton Dickinson, Heidelberg, Germany) as marker for polymorphonuclear granulocytes, polyclonal rabbit anti-actin protein, 1:200 (DAKO, Hamburg, Germany) and monoclonal anti-CD34 1:200 (BIOMOL, Hamburg, Germany), as markers for fibromyocytes, and monoclonal porcine CD31, 1:10 (DIANOVA, Hamburg, Germany), as marker for endothelial cells. The morphometric evaluation consisted of a quantitative cell analysis of the inflammatory reaction and soft-tissue reaction. The cells were counted in each of in 5 H&E-slides in 10 fields in a grid of 10 points (100x, area 0.1 mm^2) and in the interface (0-300 mm, 400x, area 625 mm^2). The parameters measured were the inflammatory infiltrate (µm), connective tissue (µm), vessels (%), macrophages (%), leukocytes (%), polymorphonuclear granulocytes (%), and fibroblasts (%), and TUNEL, Ki67 and HSP 70 expressing cells (%).

4.2.5. Statistics

All data were tested by ANOVA with the LSD modification according to Bonferroni. Statistical significance was set at $p < 0.05$.

5. RESULTS

5.1. In vitro experiments

5.1.1. Macroscopic results

Overall, the macroscopic evaluation after tissue culturing did not disclose differences in the gross appearance of the meshes. Tissue culturing was successful in 100% of the probes. No signs of infection were observed throughout the entire cultivation course.

5.1.2. Microscopic results

The tissue growth was comparable in all approaches within a test duration of 6 weeks. The testing of the biocompatibility of myoblasts, endothelial cells and fibroblasts following the addition of BioGlue® revealed unchanged tissue adherence to the mesh. Interindividual differences were not observed as concerns the growth and adherence performance after incubation with the different meshes in the investigated 10 patients.

After 6 weeks, the investigated meshes were ranked according to the descriptive/semiquantitative approach described by *Melman* et al.[33] The *Melman* score classifies tissue/cellular ingrowth on meshes as follows: none (0 point): no tissue ingrowth; fair (1 point): thin bands of fibroblasts and small collagen deposits between the mesh filaments; good (2 points): moderately thick bands of fibroblasts / collagen deposits between mesh filaments; excellent (3 points): nearly all the spaces between the mesh filaments are occupied by fibroblasts, collagen deposits and capillaries.

The ranking is shown in **Table 3a**. Interestingly, after 4 months of tissue culturing the adhesion performance was comparable for all the meshes. **Table 3b** and **Figure 4** present the ranking of the investigated native meshes and the different coating modifications.

The entire experiment was reproduced as described and a modified *Melman* score was subsequently used for the 3 different coating approaches for each patient. Analysis of the PBMC-incubated meshes indicated tissue ingrowth comparable to that for the native mesh. Interestingly, the meshes previously incubated with ATR® (Curasan Inc) and the plasma-coated meshes exhibited a slightly better performance. This trend was reproduced after 4 months of tissue culturing. All individuals displayed comparable effects of tissue ingrowth in the native state and after coating with the different blood components.

Table 3a. *In vitro* ranking of native meshes[29]

Ranking	Mesh type	Adherence score
1	Dynamesh®, FEG Textiltechnik	2.2
2	TFT Motifmesh®, ProxyBiomedical	2.0
3	Vitamesh®, ProxyBiomedical	1.6
4	UltraPro®, Hernia System Medium UHSM®, Ethicon	1.4
5	Mersilene Band, Johnson & Johnson	1.2
6	Proceed surgical mesh®, Ethicon	1.2
7	TVT PP	1.0

Table 3b. *In vitro* ranking of meshes after coating with different blood components[32]

Mesh type	Native	PBMCs	ATR	Plasma
Dynamesh®, FEG Textiltechnik	2.2	2.1	2.6	2.7
TFT Motifmesh®, ProxyBiomedical	2.0	2.0	2.3	2.7
Vitamesh®, ProxyBiomedical	1.6	1.4	1.7	1.9
UltraPro®, Hernia System Medium UHSM®, Ethicon	1.4	1.4	1.6	1.6
Mersilene Band, Johnson & Johnson	1.2	1.2	1.4	1.5
Proceed surgical mesh®, Ethicon	1.2	1.1	1.4	1.5
TVT PP	1.0	1.1	1.4	1.6

Tables 3a and 3b. *For assessment of the adherence score after 6 weeks, we evaluated each mesh with tissues from 10 different patients. After semiquantitative determination, we obtained the frequency distribution of the score results for each mesh (points/10). The scoring was based on the classification proposed by Melman et al.*[33]

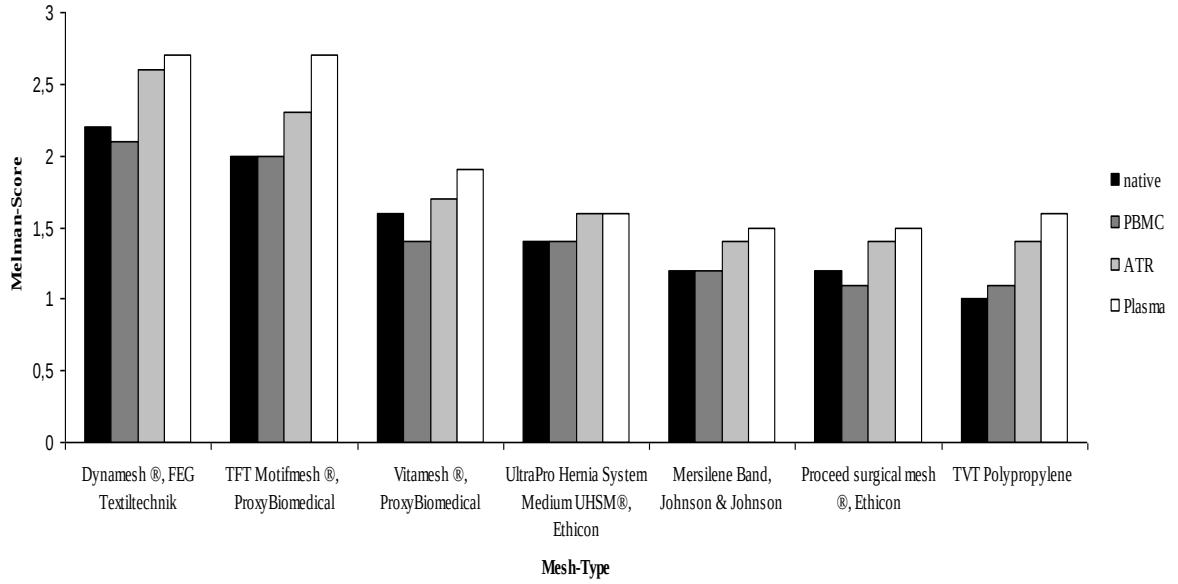


Figure 4. *Melman score comparison for different mesh coating modalities. The frequency distribution (y-axis) of the modified Melman score for each mesh is compared with the respective coating among the investigated 7 meshes.*[32]

5.2. In vivo experiments

After the surgical implantation procedure we did not see major complications in the animals during the long-term study during 24 months. Only in 1 sheep did a seroma occur, on day 3 postoperatively, which had to be drained. All the animals survived and gained weight during the investigation period. At each explantation time point, we excluded zoonoses microbiologically through vaginal, nasal and oral smears. There were no clinical infections or mesh-related complications during the follow-up. We explanted the meshes after 3, 6, 12 or 24 months. In the microscopic investigation of the different mesh reactions after explantation, the main focus was on the parameters measured for inflammatory infiltrate, connective tissue and macrophages (CD68). The respective quantifications are demonstrated in **Tables 4a-c**. For each explantation time point, we observed the same tendency in the investigated parameter (**Figures 5a-c**). High levels of connective tissue reaction and inflammatory reaction were assumed as indicative of a reduced biocompatibility. The ranking originating from the *in vitro* test system was reproducible, characterizing PVDF as the mesh (among the 3 meshes investigated) with the least FBR, scar formation and inflammatory reaction at every individual time point. Reinforced PP (UltraPro®) was in second and PP (TVT) in third position. This constant ranking was repeated throughout the entire experiment. Moreover, the modified coated versions of the 3 meshes revealed the same result at a lower level of the

respective reactions (**Figures 5a-c**). The entire experiment suggested a beneficial effect of plasma coating prior to implantation, as shown in **Figures 6a-c**. The extent of improvement remained variable in the different meshes.

Table 4a. *In vivo* course of inflammatory infiltration. Average values and standard deviations are shown for every measurement (thickness of the infiltrate in μm).

	3 months		**6 months**		**12 months**		**24 months**	
Mesh	**Native**	**Coated**	**Native**	**Coated**	**Native**	**Coated**	**Native**	**Coated**
UltraPro®	25±11	20±8	24±7	19±5	21±10	17±4	22±11	18±6
TVT	35±12	33±10	32±8	30±6	33±14	26±9	28±8	28±12
PVDF	20±9	16±4	21±9	17±8	14±9	13±6	15±6	12±2

Table 4b. *In vivo* course of connective tissue infiltration. Average values and standard deviations are shown for every measurement (thickness of the infiltrate in μm).

	3 months		**6 months**		**12 months**		**24 months**	
Mesh	**Native**	**Coated**	**Native**	**Coated**	**Native**	**Coated**	**Native**	**Coated**
UltraPro®	33±18	24±4	32±7	24±11	40±19	33±12	38±13	34±7
TVT	37±12	30±17	36±8	28±12	43±14	20±19	41±10	24±12
PVDF	25±12	19±7	22±9	23±10	22±9	17±9	24±5	19±3

Table 4c. *In vivo* course of macrophages (CD68).

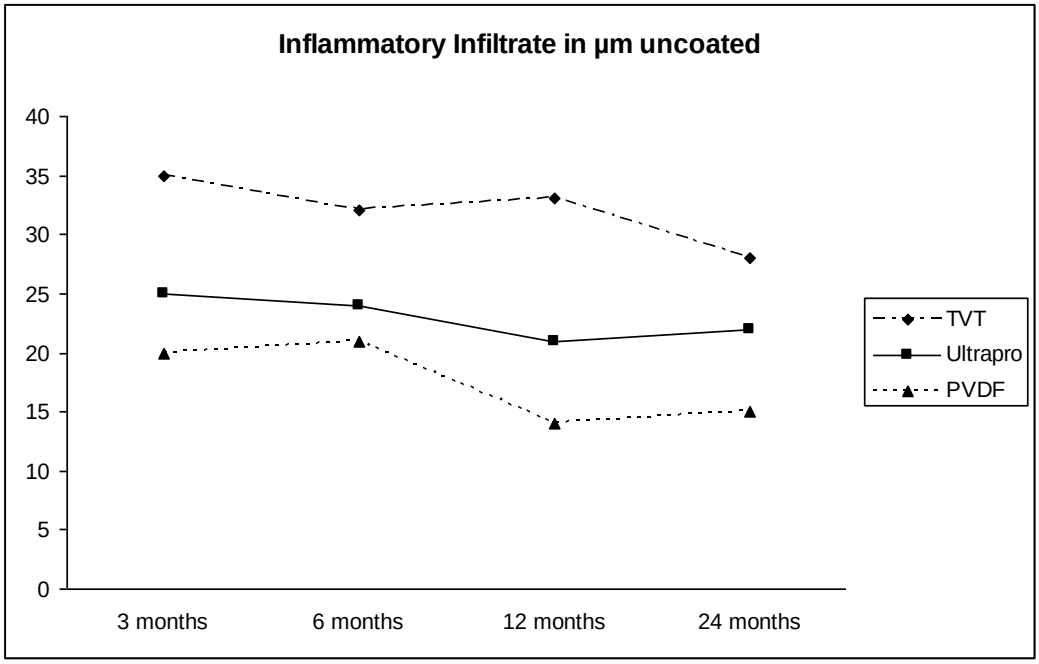
	3 months		**6 months**		**12 months**		**24 months**	
Mesh	**Native**	**Coated**	**Native**	**Coated**	**Native**	**Coated**	**Native**	**Coated**
UltraPro®	34±6	34±9	28±11	26±12	25±6	19±4	22±4	18±6
TVT	36±12	26±11	33±9	19±3	23±4	18±5	20±6	21±7
PVDF	24±4	22±8	18±7	16±3	18±8	14±2	16±5	15±1

During the ultra-short-term study, neither minor nor major complications were encountered during surgery. In addition, no macroscopic differences were observed between the native and plasma-coated meshes immediately after explantation. As in previous studies, the main focus was on parameters measured for inflammatory infiltrate, connective tissue, macrophages (CD68) and endothelial cells as markers for vascularization and myofibroblasts in the microscopic investigation of the different mesh reactions after explantation. High levels of connective tissue reaction and inflammatory reaction were assumed to be indicative of a reduced biocompatibility. **Figures 7 a-b** graphically demonstrate and relate the short-term and long-term courses of 5 important markers for early FBR determination.

Within the first 2 h after implantation, an early invasion of macrophages at a comparable extent was observed in all meshes, culminating after 120 min. The induced inflammatory reaction expressed by the extent of inflammatory infiltrate revealed the same trend, but increased slowly. Macrophage invasion was detectable after 20 min at a relatively high level of about 50%, and increased slightly up to 70%. Interestingly, the macrophage invasion was highest in the PVDF meshes, which in the long-term approach performed best with lowest chronic inflammation. The respective early inflammatory infiltrate continuously increased within the first 60 min in all the investigated meshes. However, after 120 min this trend was reversed in the PVDF meshes. In contrast for TVT and UltraPro® the inflammatory infiltrate was still increasing up to 120 min. Not surprisingly, no connective tissue was observed after 120 min. Additionally measured endothelial cells representative of vessel integration and myofibroblasts were all negative during the initial 120 min after implantation.

Two markers, representative of early FBR signs, did show relevant activity within the first 2 h after mesh implantation. For those markers, therefore, inflammatory tissue and macrophage invasion, a comparison of the coated versus the uncoated version of the respective meshes was possible, but did not indicate relevant differences.

Each mesh (coated and uncoated) was placed and investigated in 3 different positions of the torso (**Figure 3**). Differences regarding the reaction of the FBR determinants on the meshes were not observed either in the short-term or in the long-term approach when the different implant locations were compared. Further, the plasma coating did not influence the mesh performance in the different regions of the body.



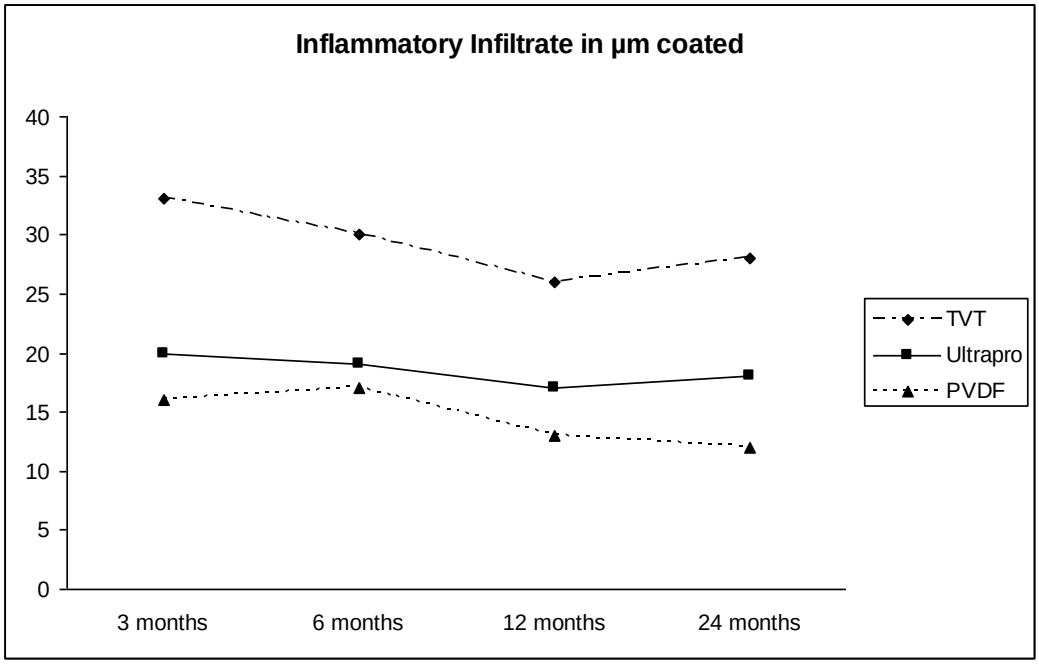
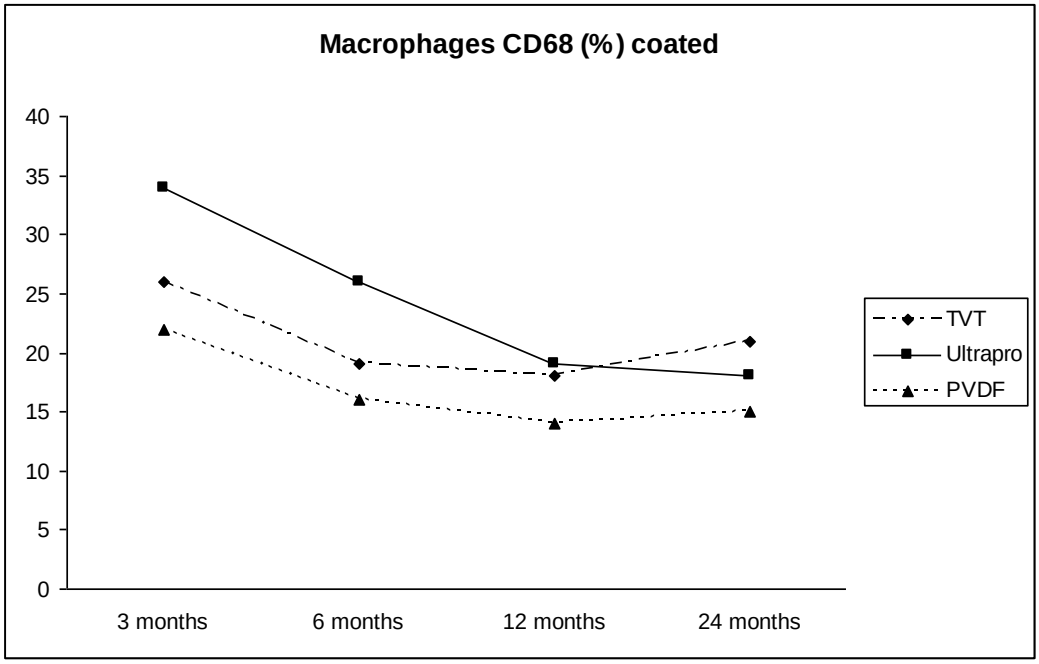


Figure 5a. *In vivo ranking and validation. In vivo ranking of PVDF, PP (TVT) and reinforced PP (UltraPro®) meshes with regard to the extent of the inflammatory reaction. The ranking is depicted for the coated and native versions of the meshes.*



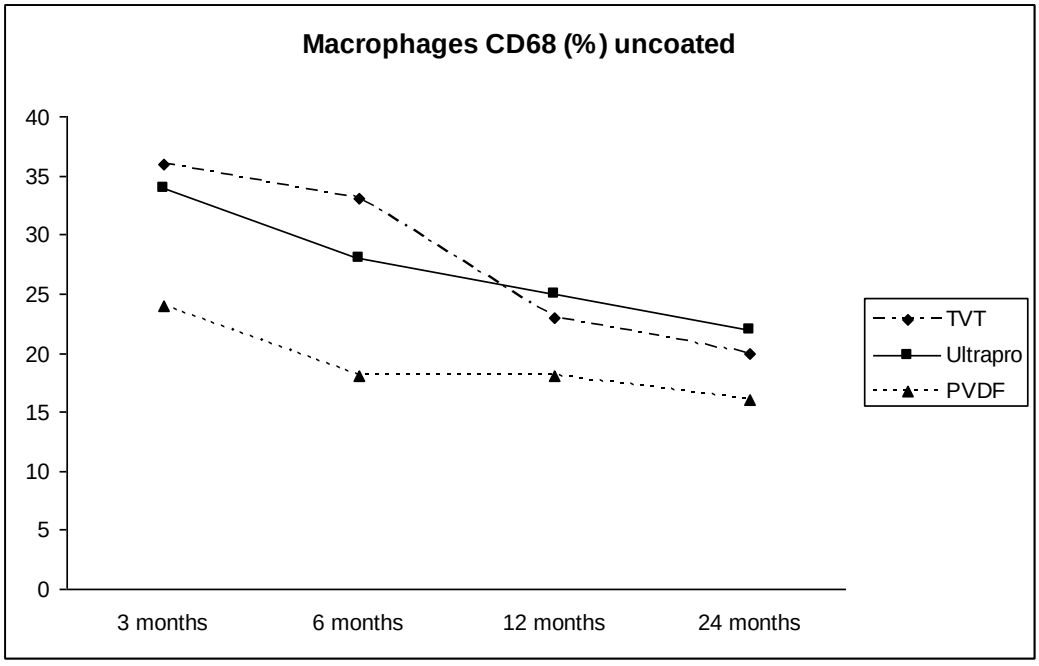
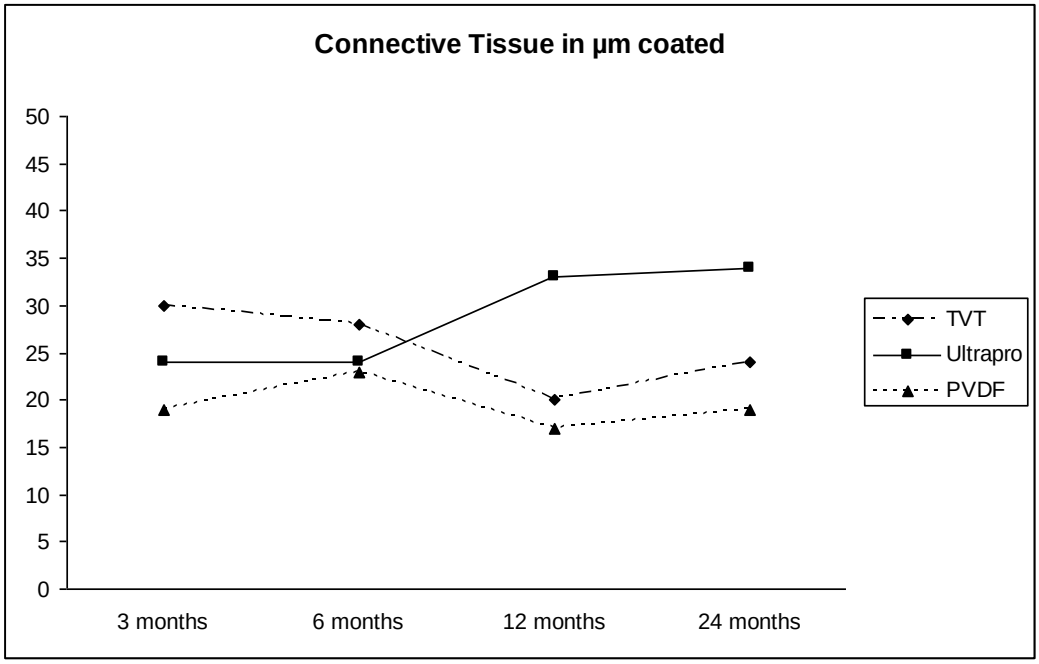


Figure 5b. *In vivo ranking and validation. In vivo ranking of PVDF, PP (TVT) and reinforced PP (UltraPro®) meshes with regard to the extent of the macrophage count. The ranking is depicted for the coated and native versions of the meshes.*



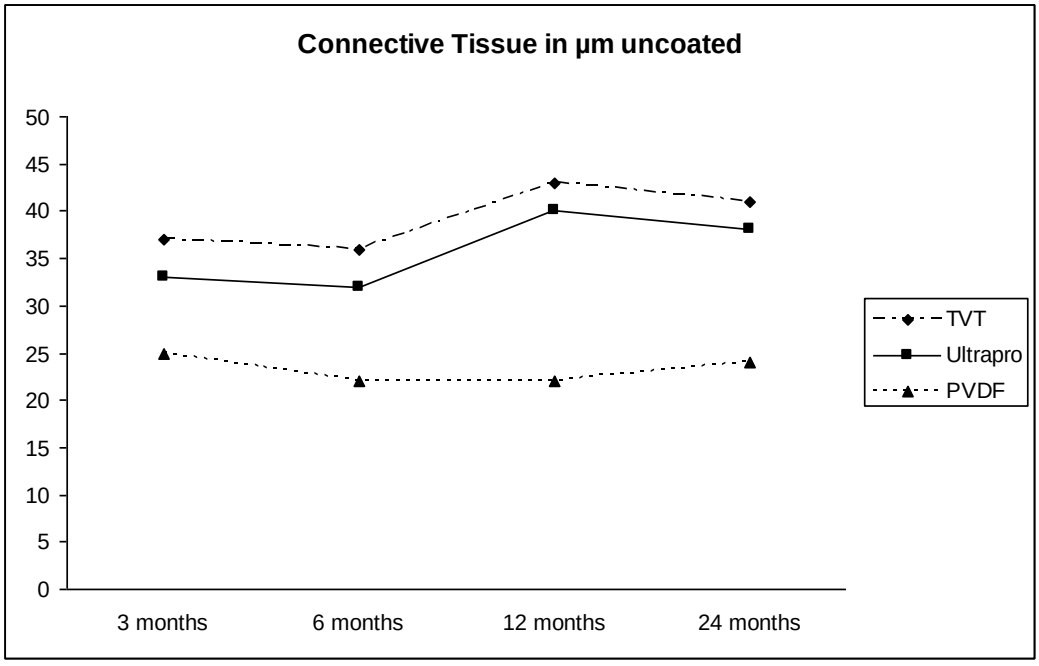
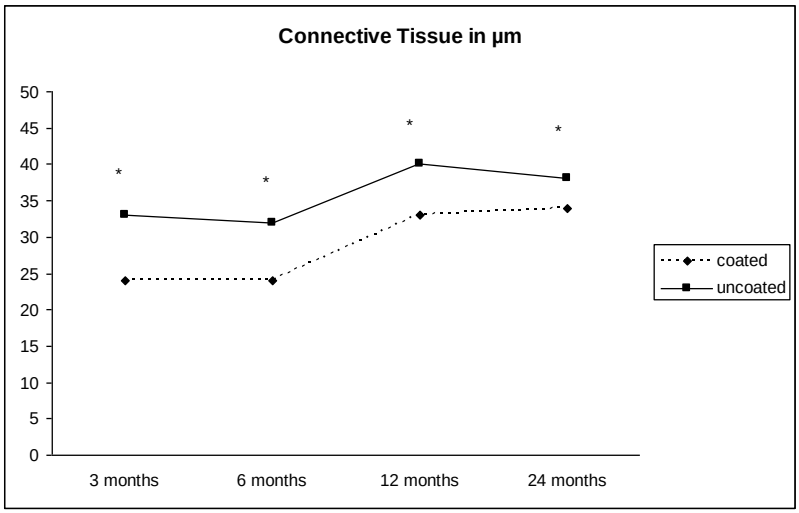
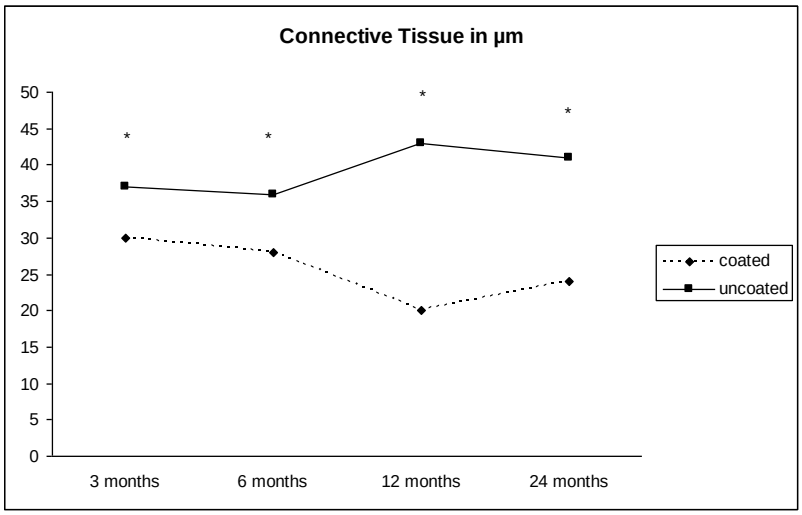


Figure 5c. *In vivo ranking and validation. In vivo ranking of PVDF, PP (TVT) and reinforced PP (UltraPro®) meshes with regard to the extent of connective tissue formation. The ranking is depicted for the coated and native versions of the meshes.*

ULTRAPRO



TVT



PVDF

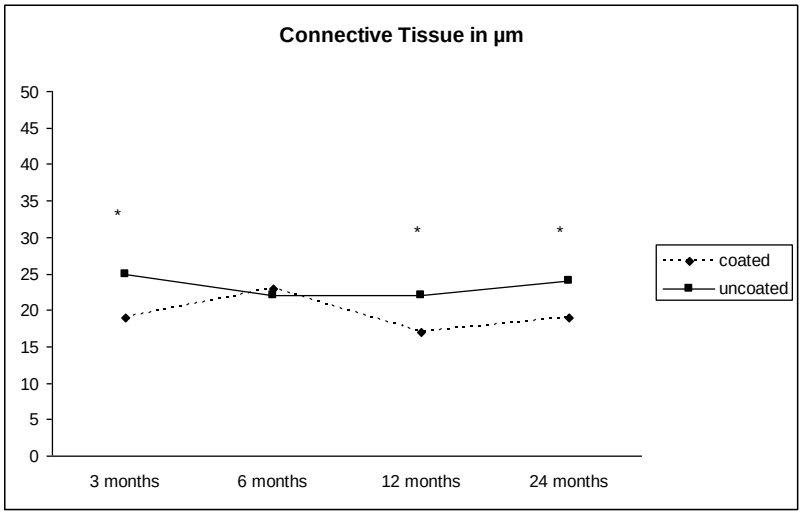
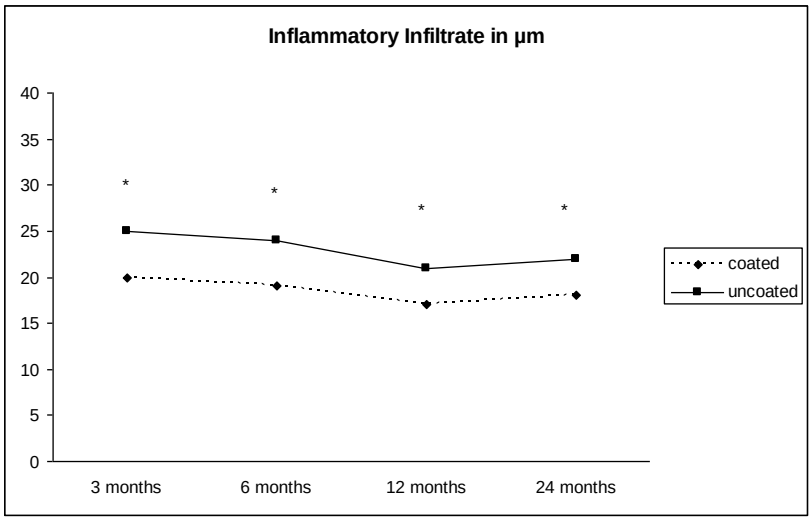
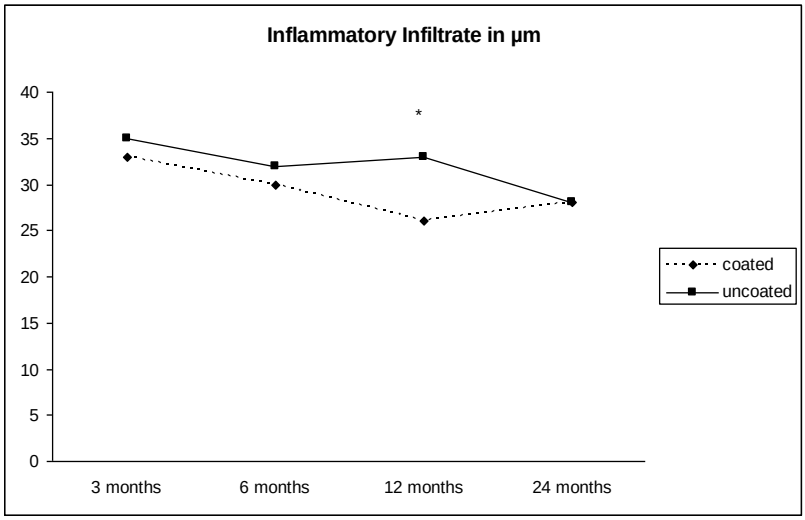


Figure 6a. *Effect of plasma coating prior to implantation. A high level of connective tissue is related to a reduced biocompatibility. Statistically significant differences in the thickness of the connective tissue are indicated by an asterisk (*), corresponding to $p < 0.05$.*

ULTRAPRO



TVT



PVDF

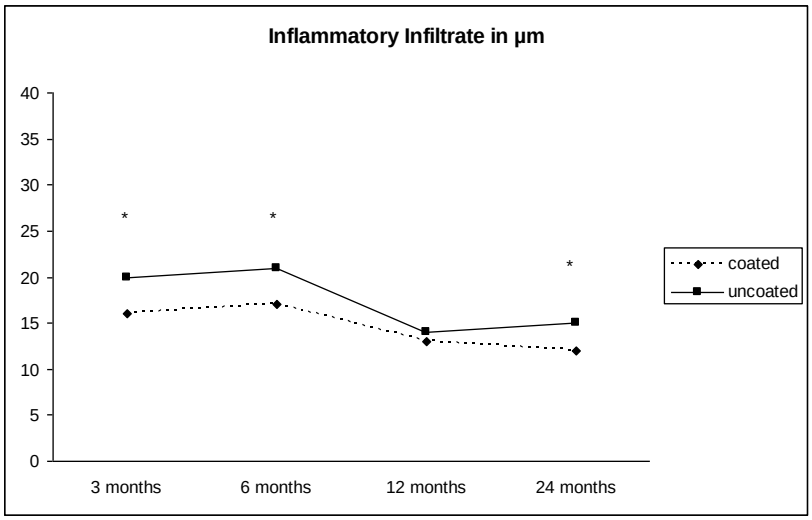
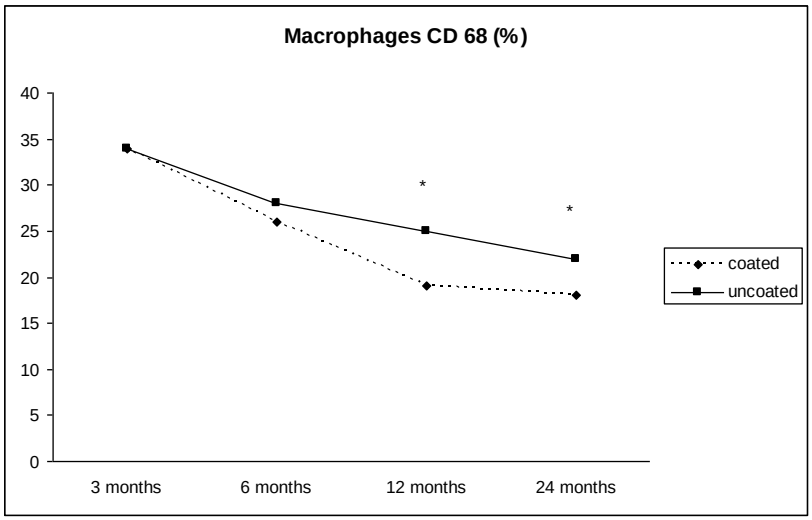
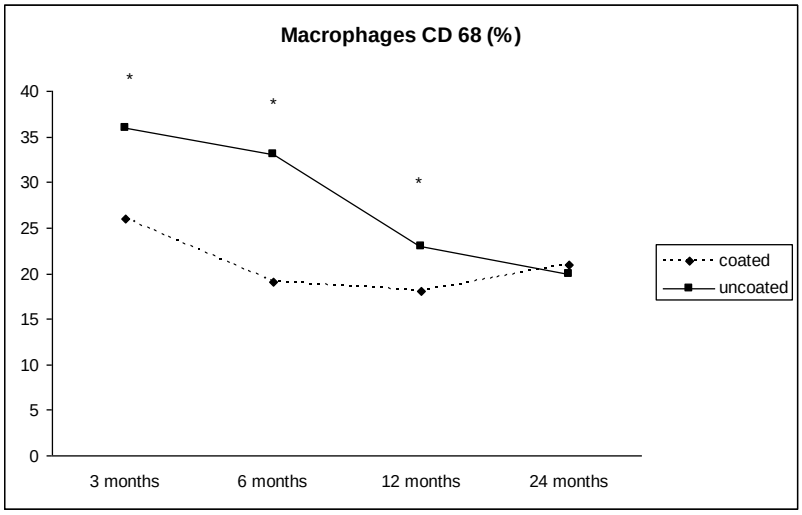


Figure 6b. *Effect of plasma coating prior to implantation. A high level of inflammatory reaction is related to reduced biocompatibility. Statistically significant differences in the thickness of the inflammatory infiltrate are indicated by an asterisk (*), corresponding to $p < 0.05$.*

ULTRAPRO



TVT



PVDF

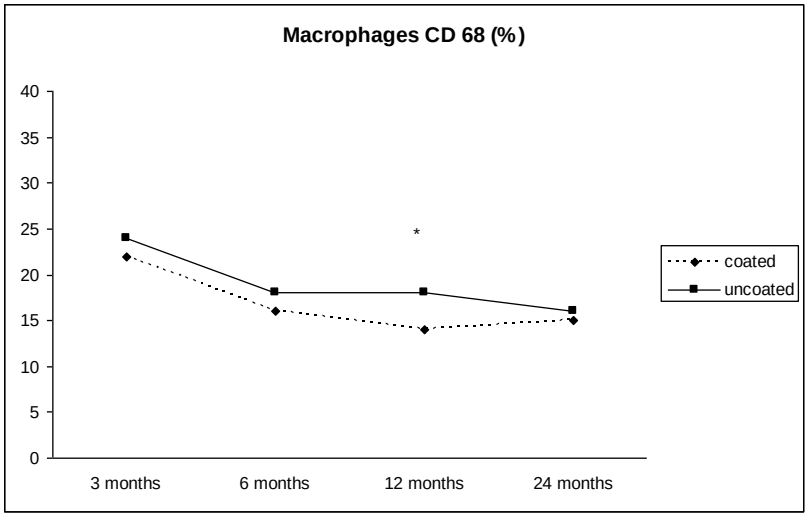


Figure 6c. *Effect of plasma coating prior to implantation. A high macrophage count is related to a reduced biocompatibility. Statistically significant differences in the percentage of the invading macrophages are indicated by an asterisk (*), corresponding to $p < 0.05$.*

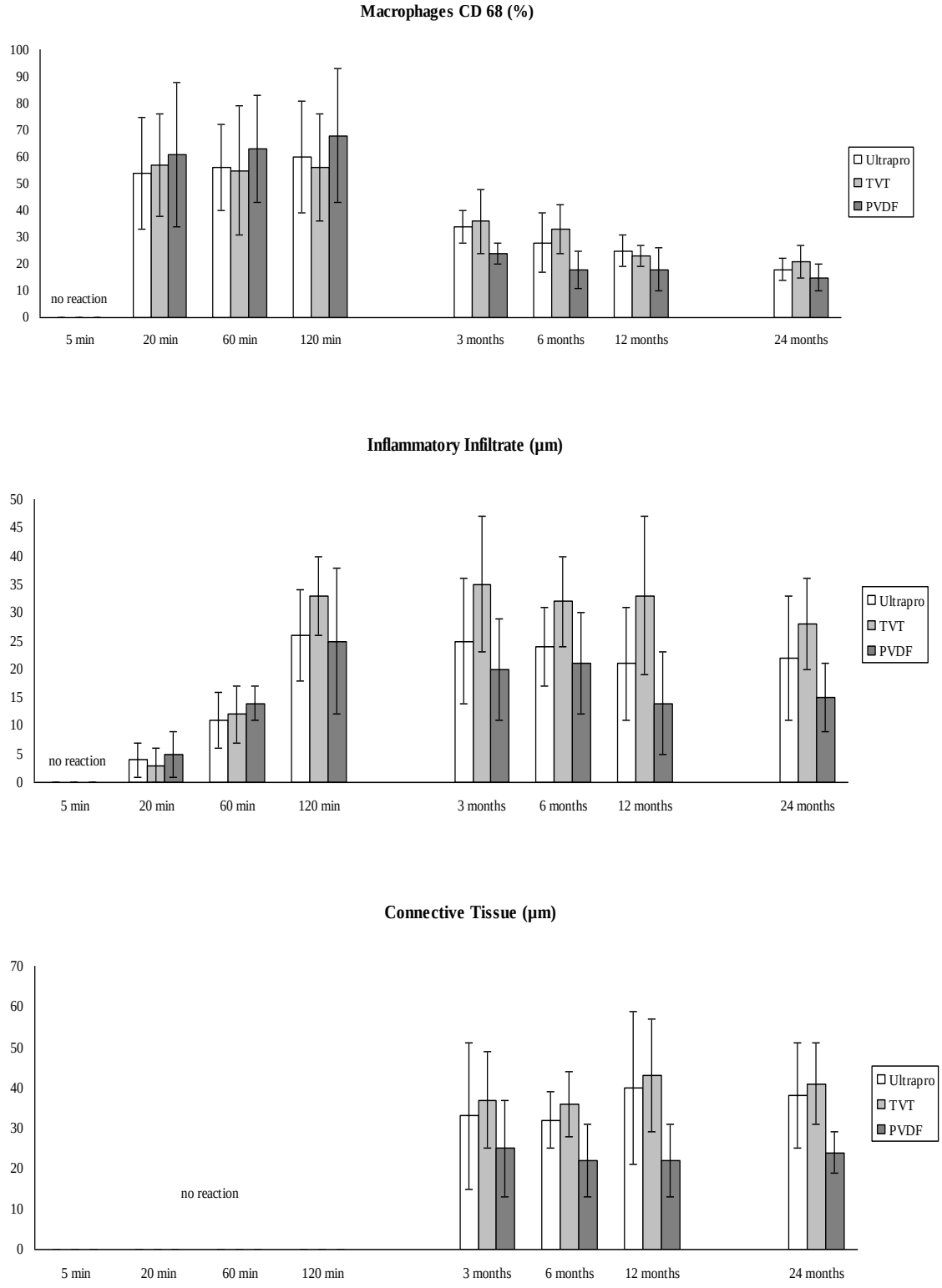


Figure 7a. *Time course of investigated biocompatibility markers, relating ultra-short-term results to long-term results (during 24 months).*

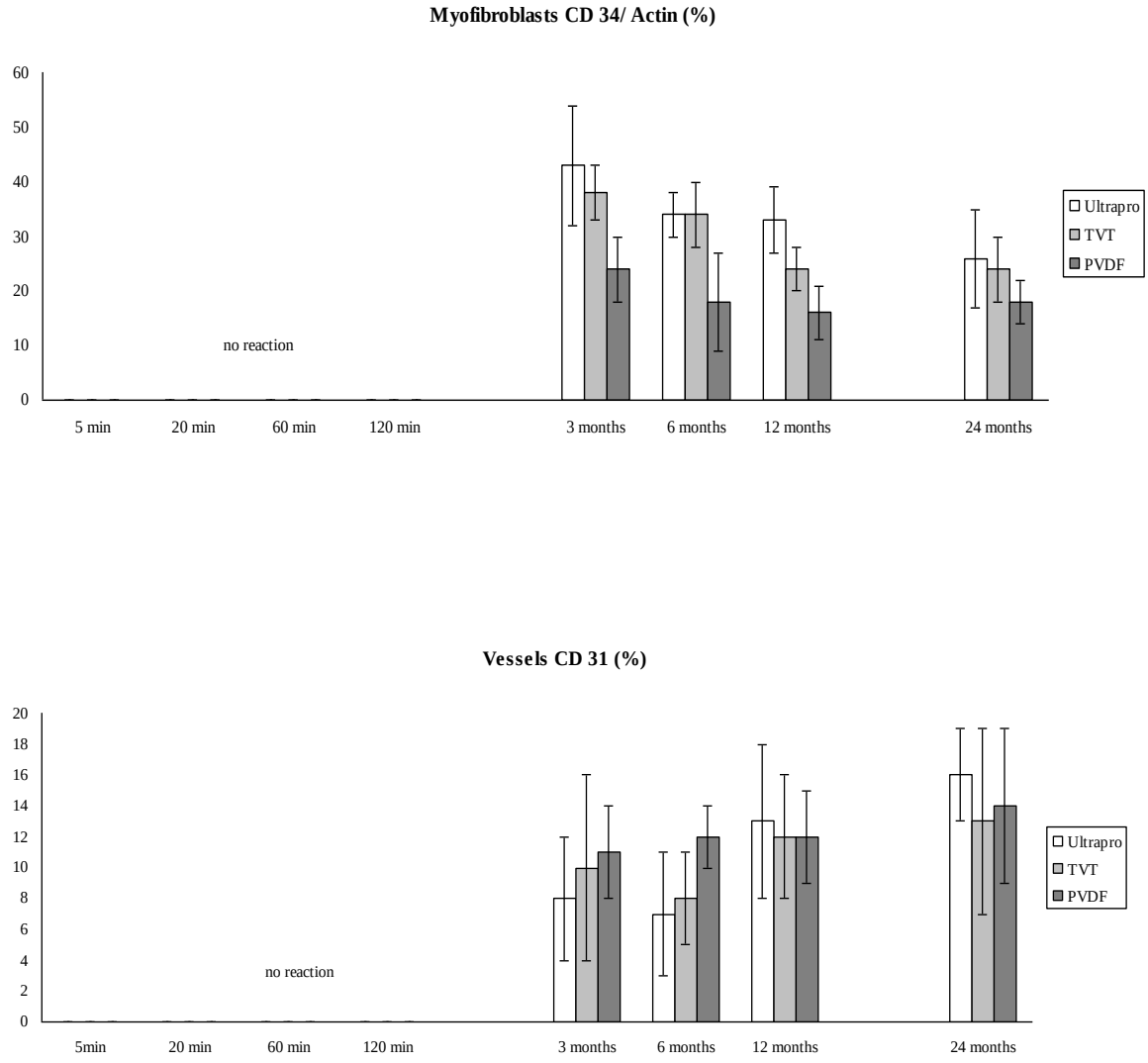


Figure 7b. *Time course of investigated biocompatibility markers, relating ultra-short-term results to long-term results (during 24 months).*

6. DISCUSSION

6.1. In vitro test system

At present, many alloplastic materials are used without proper trials and are recommended by manufacturers rather than on the basis of data arising from *in vitro* or *in vivo* experiences. The standardized classification put forward by *Amid* in 1997 is one of the few tools which allows prediction of the biocompatibility performance of a surgical mesh.[4] In that classification, importance is attributed to the pore size of the meshes as predictive of the expectable adverse event rate, suggesting that the classification should be taken into account when clinical decisions are made. Large porous meshes are currently regarded as the best tissue-integrative, with the least FBR, inflammation and fibrosis, whereas small-pore mesh modifications are associated with a significantly greater FBR and inflammation frequently related with the phenomenon of bridging, which may finally cause significant contraction or shrinkage of the mesh.[4]

The aim of Study I was to investigate whether different tissues of the pelvic floor demonstrate different *in vitro* interaction characteristics with alloplastic materials currently used as meshes in different clinical indications, *i.e*. biocompatibility features rather than mechanical characteristics. Most of the meshes investigated in the present study consist of PP as basic material. We searched for a feasible and reproducible test system that allows the assessment and comparison of meshes with regard to their *in vitro* adherence scores to different tissues, as markers of their biocompatibility.

The biocompatibility assessment of alloplastic materials through the use of appropriate cell cultures *in vitro* is a valid and accepted method which yields information about the toxicity of the investigated material, and possible effects on the metabolism and growth of the cells.[8] Langer *et al.* investigated the cellular response of human fibroblasts cultured on different PP meshes, in particular with regard to the mesh material and structure.[34] They used a method comparable to the one described here, implementing scanning electron microscopy. Their major conclusion was that the polymer surface and structure had a paramount influence on the biocompatibility of the meshes, as they identified fibroblasts preferably growing on low-weight meshes, thin filaments and mesh nodes. In contrast heavy-weight meshes were revealed to induce degenerative cell reactions resulting in a reduced biocompatibility. In contrast with Langer *et al.* we used a tissue culture approach, as our initial results with cell cultures did not reveal sufficient cell growth. Although investigation of the adherence of specific cells is useful, we consider that the co-incubation of implants with tissue clusters is comparable to *in vivo* processes. We can support the thesis of

Langer *et al.* that the microstructure of the meshes has a relevant impact on the growth and adherence behavior of cells imitating *in vivo* surroundings. Besides the presence of fibroblasts, we investigated muscle-derived and endothelial cells presenting relevant tissues of the pelvic floor. These observations resulted in a ranking of the investigated meshes as concerns their affinity for the co-incubated tissues/cells. Our ranking is in good accordance with the suggestions of *Amid*.[4] However, material features other than pore size seem to play a role for the *in vitro* performance, explaining the different scores. Moreover, it emerged reproducibly that the adherence behavior was independent of the individual patient features, thereby supporting the idea that the biological behavior of a mesh in contact with host tissues, is mostly conditioned by the structure of the biomaterial or/and its chemical composition rather than by individual host characteristics/features. PVDF, a polymer with good textile and biological properties, displayed the best adherence performance in our test system. This polymer is currently applied as PVDF-coated PP mesh for intraperitoneal-only repair.[35, 36] The main characteristics of this mesh are its macroporosity, a decreased adhesion rate to the bowel and a favorable biocompatibility *in vivo*, with low rates of inflammation and fibrosis.[5] The good performance of PVDF in our test system is therefore not surprising. To date, reported investigations of the cellular reaction on meshes in humans are based on explanted meshes after complicated postoperative courses, resulting in negatively preselected alloplastic explants.[37]

The test system presented here is far from being representative of an *in vivo* situation as the tissue culture was sterile and no probable physiological *in vivo* reaction, such as a FBR, inflammation, *etc.* was imitated. Another criticism may be aimed at the unselective investigation of the alloplastic materials. Meshes for POP, SIU or hernia repair definitely do not have identical biocompatibility/mechanical requirements. However, assumption of the adherence performance of tissue on a mesh as a possible marker of its biocompatibility seems logically independent of the respective clinical use of the implant. The test system may help to select particular alloplastic materials for further investigations, such as animal experiments, coating approaches (*in vivo* and *in vitro*), *etc.*, as we reproduce comparable extents of cell adherence on specific meshes independently of the individual patient. Thus, at least *in vitro*, specific patient features do not seem to influence the adherence performance and respective biocompatibility of the alloplastic material. Although an exaggerated FBR tissue response is assumed to be related to clinical complications, a positive role in mesh incorporation at the implant site may be triggered by bioactive mediators such as epidermal growth factor, basic fibroblast growth factor or transforming growth factor and others

produced by fibroblasts or smooth muscle cells, for example. Thus the cultivation and positive adherence of cell clusters consisting of these cell types and the respective assessment and comparison, as shown here, may be helpful in the consideration of a mesh as regards its possible tissue ingrowth and capacity to form connective tissue.

The *in vivo* behavior of a particular alloplastic material cannot be reliably extrapolated from *in vitro* studies, and appropriate *in vivo* approaches are therefore needed. The possible predictive value of these *in vitro* results with respect to cell and tissue adherence *in vivo* was the target in the following animal investigations.

6.2. In vitro plasma coating

Preclinical investigations of surgical innovations, *e.g.* meshes, in terms of bench and/or animal testing, are likely to represent a new standard requirement to confirm that engineering specifications are met and that the material and/or specific modification chosen for a mesh is sufficiently biocompatible. To date, there have been numerous reports of mesh modification approaches in order to improve their biocompatibility. The permanent character of a foreign body implant may cause persistent and increased inflammation, with ongoing collagen deposition leading to extensive fibrosis. The impaired host-acceptance of the implanted mesh is likely to appear through chronic inflammation and extensive fibrosis.[16] In order to tackle the problem of extensive FBR initiated by early local inflammation, several researchers have modified the chemical and physical properties of meshes by different coating approaches resulting in altered local reactions and tissue responses, mostly by using *in vivo* experiments. Numerous compounds have been tested so far for mesh-coating purposes, the majority of them in *in vivo* models, mostly after setting a pathological defect to be repaired by the investigated meshes.[10, 11, 38-40] Besides numerous *in vivo* experiments, *Bryan et al.* provide an *in vitro* model to facilitate the mesh choice in uncomplicated hernia repair by quantitatively determining neutrophil activation and degranulation in different mesh types.[41] Their approach represents one of the few *in vitro* assessment tools for meshes currently available in the literature. In their experiments, reactive oxygen species (ROS), released by activated neutrophils and leading to non-specific host tissue damage and potential mechanical weakening were measured on the surface of 6 different meshes. They investigated native, non-modified meshes. They concluded that the mesh structure is a greater determinant of ROS release than the chemical composition. It seems likely that their sophisticated assay could also be used for mesh assessment after different coating approaches. This would be a conclusive further development comparable to the approach

presented here, which represents an advance on the initially described *in vitro* assessment tool for native meshes.

The aim of our study was to implement and assess a facile mesh-coating procedure *in vitro* and to investigate whether the coating of meshes with autologous blood components shows different *in vitro* interaction characteristics with different tissues types as compared with native meshes. We used autologous blood components as they are relatively easy to obtain from the respective patients and contain relevant cells and substances involved in the humoral immune defense. This approach was based on the assumption that the extent to which an implanted alloplastic material elicits an acute local inflammatory response has an impact on the long-term outcome when applied *in vivo*.[42] In order to investigate cellular and non-cellular components, we separately investigated PBMC, plasma and platelets with the respective mediators. Incubation with PBMCs did not result in modification of the adherence score for the investigated tissues. This may be explained by the reduced ability of these cells to maintain permanent contact with the polymer surface of the meshes, as previously shown. In contrast, blood plasma and ATR resulted in a better adherence performance and increased biocompatibility in all meshes. An interesting observation in the current study is that all meshes previously ranked with regard to their biocompatibility performance displayed an increased score after plasma coating and maintained their position in the ranking relative compared to the other investigated meshes. This supports the thesis that coating with plasma may have an effect independent of the mesh, and, at least *in vitro*, all meshes could improve their performance, but low-ranked meshes could not increase their position relative to natively better-positioned counterparts. The thesis of *Bryan et al.* can thereby be supported: the mesh structure seems to be an important determinant of the *in vitro* performance in the native and coated configuration of a mesh.[41] Mesh-related complications are known to be related to extensive local inflammation, representing the first step of a FBR. This FBR after implantation of a mesh is assumed to be triggered by the secretion of a variety of proteins that attract inflammatory cells to migrate to the site of injury, finally leading to extracellular matrix regulation and collagen deposition. In a recent study, *Brandt et al.* investigated the effect of mesh coating (PVDF) with different substances affecting the cortisone metabolism. In their *in vivo* approach, they found that hydrocortisone and spironolactone protected from inflammatory response, ended up in smaller granuloma at the implant site of the mesh and decreased the collagen formation.[43] Their approach suggested that the respective coating approaches are a possible way to attenuate local inflammatory processes in order to reduce the FBR. This is supported by other research groups who have reported altered local cell

activation and tissue responses after modifying the chemical and/or physical properties of meshes via coating, leading to the hypothesis that the coating of polymer surfaces may be an opportunity to improve mesh integration and biocompatibility.[44] The assumption of the adherence performance of tissues on a mesh as a possible marker of its biocompatibility seems logical, independently of the respective clinical use of the implant. Although an exaggerated FBR/tissue response has been suggested to be related to clinical complications, a positive role in mesh incorporation at the implant site may be triggered by bioactive mediators such as epidermal growth factor, basic fibroblast growth factor or transforming growth factor and others produced by fibroblasts or smooth muscle cells, for instance. Thus, the cultivation and positive adherence of cell clusters consisting of these cell types and the respective assessment and comparison, as shown here, may be helpful for considering the possible tissue ingrowth and capacity of a mesh to form connective tissue. The coating of meshes with plasma and ATR appears to have a positive effect on those features.

A main limitation of this study is that no inflammatory reaction as normally cascading *in vivo* was imitated as the *in vitro* approach was sterile. In conclusion, we conducted an animal investigation (Studies III and IV) in order to validate the *in vitro* results, for both native and coated meshes.

6.3. In vivo validation

The next step in the current approach was to translate the previous *in vitro* results into *in vivo* surroundings. *In vitro* models to investigate the biocompatibility features of alloplastic materials like meshes are limited with regard to their predictibility for *in vivo* surroundings. A mesh, *per se*, is a foreign body which induces a FBR. This FBR is triggered by the initial acute phase reaction and the subsequent construction of the implant matrix, mostly conducted by the migration of fibroblasts producing glycosaminoglycans and collagen. There is ongoing debate as to which implant-induced reactions are desirable and which are not. The development of new meshes should be based on a firm understanding of the mechanisms of a FBR.[45] In our *in vivo* study, the histologic investigations for inflammatory infiltrates indicated a slight reaction associated with PVDF, which was increased in reinforced PP (UltraPro®) and even more so in PP (TVT). This reduced inflammatory reaction can be considered an expression of good biocompatibility. However, this observed postoperative sign of an inflammatory reaction was non-infectious, as counts for cells involved in the infectious immune defense, *e.g.* CD3, remained unaltered at low levels.[46] In addition, when the connective tissue investigated, the same trend was observed: PVDF exhibited the thinnest layer of connective tissue, followed by reinforced PP (UltraPro®) and PP (TVT). There was a

macrophage decrease in all meshes during the postoperative follow-up, but the highest number of macrophages was seen in the TVT meshes and the *in vitro* ranking was consistent as regards this marker. Macrophages are key mediators involved in the foreign body immune reaction, suggesting that this reaction was stronger in PP (TVT) than in the other two applied meshes. As concerns the investigated parameters, macrophage invasion, inflammatory tissue and connective tissue formation, this study confirmed the previously established *in vitro* ranking of the 3 investigated meshes repeatedly throughout the entire animal experiment, after 3, 6, 12 and 24 months. Moreover, when the meshes were modified by pre-implant coating with autologous plasma, the ranking remained constant. This supports the assumption that the recently developed tissue culture *in vitro* test system for meshes is able to predict the *in vivo* performance of the meshes. Practically, the test system helps to distinguish between meshes with good and poorer healing performances. The previously described *in vitro* test system was sterile, and thus no physiological *in vivo* reaction such as FBR or inflammation could be imitated. This indicates that the adherence ability of a mesh is crucial for subsequent FBRs or inflammatory processes which determine the *in vivo* performance of the meshes. Moreover, as in the *in vitro* approach, we did not see individual recipient features that influenced the performance of the meshes. Besides material quality issues, we assume that the process which caused FBR to the meshes must have occurred in the early period, before 3 months after implantation, since there was no further tendency to change during the following explantations.

In a recent comparable long-term study in sheep, *Zinther et al.* investigated the shrinkage of an intraperitoneal onlay mesh using a coated polyester mesh versus a covered PP mesh.[47] Besides the individual differences of the investigated meshes, they described a peak for shrinkage at 3 months, without additional shrinkage in the following 15 months, suggesting an early effect. This is in accordance with our results which indicate that an early process is responsible for the extent of a FBR and the mid- and long-term performance of an implanted mesh. This tendency is independent of the location of the mesh in the body, although its particular extent varies, depending on the site of implantation. Although those results must be confirmed in a larger series, this could be a novel approach to predict the bioperformance and integration of any available mesh, using a standardized *in vitro* experiment.

Several animal studies have been proposed and reported for the investigation of local reactions after the implantation of a mesh graft. The present study is the first experimental study conducted in sheep, with a 2-year observation period. The use of sheep as an animal

model has various advantages. The biological behavior of human cells is comparable to that of the cells in the sheep model. As compared with other large animals, sheep demonstrate a limited growth potential, while the tendency to adhesion formation (intra-abdominally) is similar to that in humans.[48, 49] In our study, we did not observe a specific reaction triggered by lymphocytes (B-lymphocytes and T-lymphocytes). Thus, it is very unlikely that the different lymphocyte status of sheep *vs.* humans may have had important influence on the *in vivo* biocompatibility performance. However, to exclude this potential bias, experiments in primates would be necessary, although very unrealistic. Given the mentioned advantages, the sheep model has the potential to serve as a template in future experimental mesh studies, in particular for the assessment of meshes in the abdominal cavity, but also other intracorporal locations. Data on adequate functional performance and material safety are currently at the focus of premarket reviews for mesh devices. Thus, preclinical investigations in terms of bench and/or animal testing are currently used to confirm that engineering specifications are met and that the material chosen for a mesh is biocompatible. Unfortunately, clinical performance data are rarely used to support clearance for meshes for whatever indication.

In the study presented here, we could demonstrate the predictive value of our recently developed *in vitro* cell culture approach for the biocompatibility assessment of meshes when translated to *in vivo* circumstances. In a second attempt, we investigated coating approaches for meshes in order to improve their biocompatibility. In preliminary experiments, mesh coating with autologous plasma was shown to reduce FBRs both *in vitro* and *in vivo*. A plasma coating seems to have a consistent improving effect on the performance of the mesh as regards connective tissue development and inflammatory local reactions at the implant site, suggesting an improved biocompatibility.

6.4. Foreign body reaction in the short term and the long term

The main purpose of our large-animal study was to investigate *in vivo* biocompatibility predictors for 3 different meshes by measuring early and long-term signs of a FBR such as macrophage invasion, and inflammatory reaction and connective tissue determination at the implant site of the meshes. By relating the ultra-short-term data to the long-term data in the same species (*i.e.* sheep), we could show that the process of determination of a FBR is defined early in the course after implantation for markers of local acute inflammation. In contrast, myofibroblast invasion, vascularization and connective tissue adhesion are not relevantly presented in the ultra-short-term course. The extent of macrophage invasion and inflammatory tissue does not relevantly increase after 120 min as compared with the values for 3 months after explantation or later. Our previously described

method to improve the biocompatibility performance of meshes *in vivo* and *in vitro* by autologous plasma coating before implantation did not have an effect on the early inflammatory events, as the respective values for inflammatory infiltrate and macrophage invasion did not differ from coated to native meshes.[1, 29, 32] However, markers such as connective tissue organization, myofibroblast invasion and endothelial cells, characteristic of vascularization, are detectable after 3 months post-implantation and show different extents in the 3 investigated meshes.

To the best of our knowledge, our results reflect the longest combined short and long-term *in vivo* approach to the investigation of biocompatibility issues on meshes. In addition, no ultra-short-term investigations *in vivo* have ever been reported so far, as most of the currently available studies investigated effects on meshes at the earliest after 7-21 days.[50] It has been shown that an acute inflammatory reaction occurs at 7 days after implantation of a mesh, dominated by macrophage invasion.[51] Over time, this early inflammatory process transforms into a chronic, at times granulomatous reaction, promoting wound healing, but also forming small granulomas.[16] It is known that the extent of collagen formation may vary during this process, whereas a severe inflammatory reaction, with disordered fibrin and collagen deposition, is likely to compromise the integration process and functional outcome. In an investigation of prolene and a porcine dermal collagen implant (Pelvicol®), *Zheng et al.* identified a first acute-phase reaction after 48 h, peaking on day 7-14.[51] Our data add ultra-short-term information, suggesting that this reaction starts even earlier in the course, after a matter of minutes. *Zheng et al.* described that the acute reaction diminished and finally reached negligible levels by 90 days, which can be partially supported in our current study.

FBRs to alloplastic mesh material are primarily induced by inflammatory cells such as macrophages and T-lymphocytes.[52, 53] Macrophages have a critical role in acute inflammation and early vascularization, and also in the subsequent chronic phase of the host response, as they are known to be capable of differentiating toward two pathways. This M1/M2 polarization enhances macrophages, leading to an immediate and/or persistent inflammation or to a constructive remodeling and new tissue generation.[54] However, this polarization was not investigated in the current study. From the aspect of wound healing, it is known that CD68-positive macrophages reach their maximum level on the second day after injury and slowly decline thereafter.[55] We have shown that high percentages of CD68-positive macrophages are detectable on the meshes after only minutes or hours. This could be of interest when investigating and developing mesh modification strategies to influence this early acute reaction. A previously developed plasma-coating strategy to optimize the

biocompatibility of meshes does not seem to influence this early inflammatory reaction and inflammatory infiltrate formation, but rather to influence mid- and long-term processes which lead to neovascularization and collagen fiber organization. It has been found that premature type III collagen is predominantly synthesized in the early phases of wound healing and in the presence of inflammatory cells.[56] This type III collagen is then replaced by highly cross-linked and stable type I collagen later after implantation. Delayed wound healing and immature scar development due to persistent chronic inflammation may be predicted by a lowered type I/III collagen ratio.[37, 57] A favorable type I/III collagen ratio is known to improve biocompatibility and can be positively influenced by the pre-implantation of mesh modifications such as a gentamycin coating.[39] As concerns the results of the present study, an increase in inflammatory infiltrate was revealed for all 3 meshes. After 120 min, PVDF was observed to increase considerably more slowly than TVT and UltraPro®. The previously shown good long-term biocompatibility performance of PVDF may hypothetically be triggered by an early decrease of the acute inflammatory reaction and subsequent modification of the micro-environment meshes, leading to an improved type I/III collagen ratio, for instance. We did not observe a direct influence of the localization of the implanted mesh in either the body in the short- or the long-term study as regards the reproducibility for the coated and uncoated meshes. Although the localizations were chosen to cover different structural parts of the body with different immunologic potentials and physical/mechanical strains, the localization does not seem to be of utmost importance for the biocompatibility performance of a mesh *in vivo*.

6.5. The project with regard to the IDEAL recommendations and future perspectives

A plethora of commercially available meshes currently make the decision as to which mesh to apply very difficult. Two FDA warnings in 2008 and 2011 reported more than 3,500 severe adverse events after mesh applications, mostly in POP and SIU patients. As a consequence, the FDA recommended the consideration of regulatory changes, including an upgrading in risk classifications for meshes, clinical studies to address the risks and benefits of meshes and expanded the post-market monitoring of device performance.[58] Our preclinical *in vivo* study was initially inspired by the first FDA warning of unexpected and severe adverse events during the use of mesh devices.[20] We raised the question of whether the performance of a mesh would be predictable prior to its implantation in order to reduce the probability of unexpected mid- and long-term events as reported and complemented in 2011.[21] Although we did not selectively investigate meshes for the indications reported as POP and SIU, our system (*in vivo* and *in vitro*) may easily be used with every available mesh.

However, in a considerably narrow time frame as a reaction to the first FDA warning, we developed an *in vitro* approach, a subsequent animal study, and are now translating our results into a clinical trial. As a first step with this regard, we have analyzed and classified specific complications after vaginal mesh implantation approaches for prolapse repair.[59] This analysis comprises the basis for a currently ongoing pilot study which is investigating the feasibility of the autologous plasma coating of meshes prior to implantation in humans. Thus, on the basis of the results presented in this work, we are applying for the first time plasma-coated meshes in the human setting (*Barski, Gerullis, Otto et al., 2014, unpublished data*). As a next and most important step, a prospective randomized trial, possibly proving the positive effect of the plasma coating on the biocompatibility and morbidity outcome, is planned on the basis of the positive preliminary results. Both studies would fulfill stage 3/A of the IDEAL classification and consistently sustain the initially chosen way of implementing this new technology of plasma coating following IDEAL.

In addition, and in order to provide the final stage 4/L step of the IDEAL approach, we are already creating and establishing an online platform for registration and outcome measurement of mesh application with clear definitions and classifications, which will be offered to the uro-gynecological community. We are therefore using well-described definitions for risk factors and possible complications based on ICS-IUGA standards in terminology and guidelines *(Barski, Gerullis, Otto et al., 2014, unpublished data).*

These steps conform to the IDEAL recommendations and show how surgical research may be concluded (independently of any result) when strictly driven following standardized recommendations. *McCulloch et al.* specified recommendations concerning IDEAL for the field of urology.[60] Although not mentioned in their review, we would add mesh-implementing procedures for an interventional option as a topic of current controversy and debate in urology/uro-gynecology, not only for safety purposes, but also for effectiveness considerations.[61-64] In conclusion, the current work displays the experimental *in vitro* and *in vivo* basis for the surgical innovation of a plasma coating on surgical meshes. The already completed and all planned stages of this innovation strictly follow the IDEAL recommendations.

7. SUMMARY OF FINDINGS

1. We have developed a manufacturer-independent *in vitro* test system for the adherence performance of tissue clusters (fibroblasts, endothelial cells and muscle-derived cells) as a marker for the biocompatibility of commercially available meshes. In this test system, we established a repeatable ranking of meshes with regard to their biocompatibility. The adherence behavior was independent of the individual patient features, suggesting that the biological behavior of a mesh is probably conditioned by the structure of the biomaterial or/and its chemical composition rather than by individual host characteristics/features.

2. The *in vitro* test system is a feasible pattern for the investigation of different coating strategies of meshes. The coating of meshes prior to cultivation with PBMCs, for instance, did not affect the adherence score, whereas coating with platelets and blood plasma increased the score, suggesting an improved biocompatibility *in vitro*. The plasma coating displayed the greatest potential to improve the *in vitro* adherence score. The previous ranking of the native meshes remained consistent after coating, but was established at a higher level.

3. In a long-term animal study in sheep, we demonstrated that the developed *in vitro* test system for the biocompatibility of meshes may predict the *in vivo* performance of the meshes. This effect is independent of the location of the mesh in the body, although its particular extent varies depending on the site of implantation. The coating of meshes with autologous plasma prior to implantation seems to have a positive effect on the biocompatibility of meshes *in vivo*.

4. Investigations of the ultra-short-term determinants of the FBR at the implant site *in vivo* revealed that the local inflammatory reaction is an early and susceptible event after mesh implantation. It cannot be influenced by prior plasma coating and does not depend on the localization of implantation.

The development of this surgical innovation (the plasma coating of meshes prior to implantation) strictly followed the IDEAL recommendations at every step in order to ensure comparability and transparency.

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