Biotechnologies, Biosciences and Surgical Technologies, XXXI cycle

PhD student: PIERO G. BRUNI MD

(reg. nr. 728033)

"Artificial vs biological meshes: can in vitro cellular responses predict the outcome in patients?"

Scientific Coordinator: prof. G. Campanelli

Index

Background

Prosthetic abdominal wall hernia surgery is a very common procedure (1) (2). Nowadays, about one million prosthesis per year for abdominal wall defect repair are used worldwide (3). Since the first description of a mesh use for abdominal wall repairing (4) plenty of new materials have been developed and introduced in the market, allowing a considerable reduction of recurrence long-term rates comparing to previous traditional surgical techniques (suture repair alone).
Over the past 20 years, implantable surgical mesh products used for abdominal wall reconstruction have evolved in an attempt to improve mesh-related repairs.
Despite the increasing number of implantations and the improvement in material engineering, our knowledge about the tissue response to implanted meshes in human and their long-term biocompatibility is still lacking, from the moment that it has been related to many adverse side effects such as seromas, infection and chronic pain (5) (6).
The consequences of synthetic mesh failure can be catastrophic especially in complex hernia repair, including contaminated or clean–contaminated tissue, bowel or fistula exposition and significant loss of domain (6). The use of synthetic meshes in all these cases can be associated with infection, significant adhesions to intra-abdominal organs, leading to obstruction, erosion and fistula formation (7). Infected synthetic meshes are difficult to treat, frequently require surgery to explant, and may leave the patient with a colonized defect requiring prolonged treatment (8).
Bioprosthetic mesh materials clearly have certain advantages over other implantable mesh materials in select indications. Appropriate patient selection and surgical technique are critical to the successful use of bioprosthetic materials for abdominal wall repair.
Biologic meshes are promoted for repair of complex hernias, as these meshes become incorporated into the wound, acting as a scaffold for tissue repair leading to a strong, well-healed, vascularized wound (9). Due to the nature of biologics, the adhesions associated with synthetic mesh should not occur and vascularization allows delivery of immune cells and antibiotic (8).
But even biologic grafts are not free from failure: inadequate mesh incorporation or degradation of implanted meshes are reported (10) (11); their use is still debated, due to high cost to benefit ratio.
Retrospective consideration of biologic mesh efficacy is compromised due to the compounding variables induced throughout their application: the variety of available products, the techniques used to repair hernias (onlay, inlay, sublay, component separation) the complex nature of hernias being repaired, the life style of the patient, the eventually presence of co-pathologies, BMI (12) (13). To date, in literature it is not possible to find some homogeneous indications on usage, reasons to choose one type with respect to another or specific clinical indications.
If we could outline a biomolecular response scheme with respect to the different meshes it would afterwards be possible to understand if the body response could be foreseen and managed proactively rather than only reactively and also try to tailor therapies in case of secondary infections or adherences. Moreover, this type of knowledge can be exploited to optimize pretreatments both for artificial and biological meshes. For instance, it can be used to understand if and how the effects of cross-linking of a biological mesh alters the inflammatory responses or if a specific coating enhances fibroblast invasion or macrophage polarization and chemoattraction.
Without this knowledge, the improvement of mesh use will be mainly driven by ex post studies, that are first based on human trials, very long, extremely costly and always not optimized in terms of homogeneity of data samples. Other studies use animal models, procedure that present again elevated costs and drawbacks both from the ethical point of view and in terms of translation to human application. Any tool or set of information that can help to foresee an advantage in a specific mesh usage in a specific setting or in a technical change of a specific mesh can provide a very large advantage in health gain and economical perspective.

In the first part of this paper a review of all more recent data available about the biological graft behavior and interaction with tissue is presented. In the second part results from our laboratories

about interaction between different matrices (synthetic and biological) used in abdominal wall repair surgery and a fibroblast colture are reported. In the third part an initial experiment in vivo is mentioned, focused on a morphologic analyses by SEM and TEM of the same different prosthetic materials used in vitro after underskin implantation in the leech.
Finally, in the fourth part, our clinical experience with biological meshes is reviewed.

Literature review

The ideal mesh

The characteristics of ideal hernia repair materials are perpetually refined.
The qualities described by Cumberland and Scales, and Hamer-Hodges and Scott, are as follows: non-carcinogenic, chemically inert, resistant to mechanical strain, sterile, unresponsive to body and tissue fluids, able to limit foreign-body reaction, modifiable to defect size, and non-allergenic (14). Required characteristics applied specifically in the instance of biologic meshes include resistance to infection, anti-adhesive, and the capacity to act like native tissue (8). Additionally, an ideal material should be associated with very little surgical morbidity, such as seroma, easy to handle in open and laparoscopic instances, and cost effective.

Biological graft

Biologic graft material composed of purified porcine small intestinal submucosa was first introduced to the United States in 1998, as an alternative to synthetic biomaterials. Later, biological meshes derived from other tissue of origin had been presented on the market. These meshes are composed of extracellular matrix (ECM) collagen, fibronectin, associated glycosaminoglycans, and growth factors (15) (16) (17) (18).
Biological meshes are considered as a scaffold for the binding of growth factor and other cellular elements for the healing response. The subsequent healing response and strength are dependent on ingrowth from the patient's cells and blood vessels into the ECM of the biologic graft.
The balance between ECM synthesis and degradation contributes to the ultimate success of the hernia repair.
The current biological meshes present on the market (tab. 1) differ in the mammalian source (animal or human), tissue of origin (dermal, pericardial, bladder or intestinal submucosa), as well as their method of processing (cross-linked or not cross-linked) and sterilization.
All of these differences may lead to differences in the healing process and thus clinical outcome.
Mammalian source may be considered when choosing among the various biological prosthesis available. Human cadaveric tissues offer the advantage of using allograft (within species) sourcing and thus lacking interspecies rejection risk. The source of such tissues is donor dependent, with variability in composition, health, thickness, and age of the tissue.
Alternatively, animals can be raised to precise specifications to achieve a more consistent product. The risk of allergic response to their ECM is low because of the high homology with similar human proteins. With nonhuman tissues, the risk of tissue rejection remains despite decellularization, as does the rare possibility of disease transmission.
Biological grafts vary in their tissue of origin. The dermis remains the preferred tissue source, though products made from alternative tissues, such as the pericardium, stomach, bladder, and intestinal submucosa, are also available.
The purported function of biologics is to serve as a regenerative framework that supports matrix remodeling and new collagen deposition (19) (20). While tissue source may certainly affect how a given mesh is reacted upon by the recipient, differences in tissue reaction are likely a result of the different methods of processing, decellularization, and sterilization used. Manufacturers utilize various proprietary methods and processing solvents that likely influence the innate biochemical and biomolecular structure of the collagen scaffold. Subsequently, these matrix alterations likely influence "foreign body" recognition and antigen presentation. The resulting processing changes likely influence the biocompatibility, foreign body response, and immunogenic potential of each implant. In fact, it has been suggested that the manufacturing process for each mesh may be more critical to implant function than the source and the species from which the mesh is derived (21).

Table 1 Bioactive prosthetic materials

Mesh name	Vendor	Source	Cross-linking	Sterilization
Alloderm	LifeCell-Acelity	Human dermis	No	None
AlloMax	Bard/Davol	Human dermis	No	Gamma irradiation
CollaMend	Bard/Davol	Porcine dermis	Yes	Ethylene oxide
FlexHD	Ethicon	Human dermis	No	None
FortaGen	Organogenesis	Porcine intestine	Yes	Gamma irradiation
Fortiva	Rti Surgical	Porcine dermis	No	Gamma irradiation
MatriStem	ACell	Porcine bladder	No	E-beam
Peri-Guard	Synovis	Bovine pericardium	Yes	Liquid alcohol
Permacol	Covidien	Porcine dermis	Yes	Gamma irradiation
Strattice	LifeCell-Acelity	Porcine dermis	No	E-beam
SurgiMend	TEI Biosciences	Fetal bovine dermis	No	Ethylene oxide
Surgisis-Biodesign	Cook Medical	Porcine intestine	No	Ethylene oxide
Tutopatch	Tutogen Medical	Bovine pericardium	No	Gamma irradiation
Veritas	Synovis	Bovine pericardium	No	E-beam
XCM Biologic Tissue Matrix	Synthes	Porcine dermis	No	Gamma irradiation
XenMatrix	Bard/Davol	Porcine dermis	No	E-beam

Collagen cross-linking

Of all the various processing techniques used by the manufacturers, collagen cross-linking appears likely to have the most profound effect on tissue responses to biologic meshes. By using hexamethylene diisocyanate, carbodiimide, glutaraldehyde, or photo-oxidizing agents (22) (23), intentional cross-linking is utilized to prolong the lifespan of the mesh (24), using processing techniques that add to the three-dimensional structure of the collagen to, essentially, mechanically strengthen the matrix and impede degradation by collagenase.

Even nonintentionally cross-linked products may undergo molecular structural changes, such as collagen cross-linking, from gamma irradiation during the sterilization process (25). In addition, incomplete removal of chemical cross-linking agents could result in cytotoxicity from residues leaching from the mesh itself, which may induce prolonged toxic effects and heightened cellular responses (22). While clinical circumstances requiring long-term tissue reinforcement may provide some utility for a cross-linked graft, numerous investigators have recently reported disadvantages of chemical cross-linking in both translational animal models and the clinical setting. These complications included acute mechanical failure of the mesh, degradation of the mesh, and poor integration of the mesh. Poor mesh integration is a result of poor angiogenesis into the material, which can lead to encapsulation or prolonged inflammatory response characterized by foreign body giant cell reaction (26) (27) (28) (11) (29).

Mesh integration

Biologic mesh integration remains an important and desirable outcome. Unfortunately, this process is poorly understood and is often difficult to qualify and quantify/measure. It appears that a cascade of events follows mesh implantation. After the placement of mesh into the host, an acute inflammatory response seems to take place. This is a necessary event in wound healing, and

obviously, it is highly influential in biologic mesh performance. The process of ingrowth begins with mononuclear cell (macrophages and mast cell) penetration into a mesh scaffold. While meshes with diminished biocompatibility do not allow for such neocellularity, grafts that are positively recognized by a host will have host cells easily migrate from the periphery of the mesh inward. This step is often simultaneous with new blood vessel proliferation within the graft. Once mononuclear cells populate the graft, the typical sequence of wound healing events likely takes place. Mononuclear cells secrete cytokines and other signaling factors to attract fibroblasts. Once fibroblasts arrive, new collagen synthesis and deposition take place. Importantly, this process has to occur not only at the mesh/host interface but also within the graft itself. This would predispose a biologic graft for ingrowth, incorporation, and new collagen deposition within the mesh (30).

Foreign body response

Inflammation appears to be a common component of host response to implanted biologic prosthetics (21) (29) (24) (31). This reaction may either aid in the integration of the mesh via normal wound healing mechanisms or induce a disproportionate inflammatory response. Such an exaggerated reaction will likely result in excessive scarring, graft encapsulation, and/or degradation (10) (21) (29). The balance between appropriate wound healing and detrimental sequelae is largely controlled by cytokines, growth factors, and other chemical signaling molecules produced by host macrophages at the site of host/mesh interface. Orenstein et al. were the first to evaluate the immunogenic potential of various human-derived and porcine-derived biologic meshes in vitro (29) (32). With regard to the former, AlloDerm appeared to induce the smallest degree of cytokine production, indicative of superior biocompatibility (32). With regard to porcine meshes, non-cross-linked porcine dermis mesh and, to a lesser degree, porcine intestinal submucosa–derived mesh, were associated with a markedly diminished cytokine production as compared with the cross-linked porcine dermis materials. While the exact clinical importance of the excessive macrophage activation in vitro is unclear, that early evidence of adverse effects of chemical cross-linking has subsequently been corroborated by a number of in vivo studies (10) (21) (28) (33).
Most recently, Petter-Puchner et al. reported a pronounced foreign body response to intraperitoneal implantation of Collamend and Surgisis in rats. Both meshes were noted to be surrounded by a broad rim of foreign body giant cells and granulomas (27). In contrast, another recent industry-sponsored study surprisingly found no evidence of inflammatory or immune response to Permacol (10).

Neocellurization and Neovascularization

Early cellular and vascular infiltration of a biologic matrix is critical for mesh integration. Monocyte/macrophage penetration of the graft from the surface inward is paramount for fibroblast proliferation and new collagen deposition. In the absence of angiogenesis, remodeling will not occur, and the matrix will be replaced by scar. Butler et al. found that non-cross-linked porcine dermis promoted early cellular and vascular infiltration and likely contributed to a stronger mesh/musculofascia interface (28). Xu et al. reported that functional blood vessels paralleled host cell repopulation with clearly delineated channels lined with endothelial cells in human dermis by 1 month after implantation in primates (31). These findings were confirmed more recently in a sublay biologic mesh study in rats (10). The authors found that the AlloDerm group was associated with 100 percent neocellularity by 3 months after implantation. Neovascularization was clearly noted to support the cells. Finally, a normal, nondenatured collagen pattern was seen, indicative of remodeling and new collagen deposition (10). Of note, these findings were not seen in the cross-linked porcine dermis groups. It is also important to point out, however, that Deeken et al. have recently suggested that such early drawbacks of cross-linked meshes may not be significant in the long term (24).

They report that although cross-linking differentiated biologic meshes with regard to cellular infiltration and neovascularization early on, those histologic features were no longer affected by cross-linking at a 1-year time point. While these isolated results are intriguing, it is unclear whether the findings by Deeken et al. are true representations of what happens in humans or just one of the limitations of long-term ventral hernia/biologic mesh investigation in resilient animal models such as the minipig. Another interesting angle of biologic mesh utilization was investigated in a recent study by Petter-Puchner et al. (27). They used various biologic meshes (cross-linked and not cross-linked) with rows of perforations. They discovered that neovascularization was enhanced through the perforation, even though perforations had no positive effect on integration and ingrowth.

Matrix Remodeling

The final and most important step in biologic mesh placement is graft integration and remodeling with new collagen deposition and tissue regeneration. During this process, the implanted mesh is often resorbed by the host. Melman et al. have suggested that when scaffold degradation is accompanied by cellular infiltration, extracellular matrix deposition, and neovascularization, it can be viewed as remodeling (34). At times, however, when extracellular matrix deposition/neovascularization does not occur, mesh is likely replaced by a scar with a resultant detriment to a hernia repair. One of the other key factors that influence remodeling may be the rate of scaffold degradation (34). Almost uniform failure of absorbable meshes may be due to a fairly rapid degradation of the graft without proper support for new extracellular matrix components deposition. In fact, a gradual remodeling of an implanted tissue graft seems to be essential for abdominal wall repair because degradation or absorption of a scaffold not balanced with deposition of new collagen would predispose to mesh failure (31)

Deeken et al. reported that non-cross-linked grafts exhibited more favorable remodeling characteristics. However, remodeling in their study was not associated with stronger reinforcement of native tissue repairs in the long term (24). These paradoxical results may be a consequence of the animal model used. Another recent study revealed essential lack of matrix absorption and absence of remodeling of cross-linked graft 6 months after implantation (10). Given its lack of integration into the host and likely resultant fibrous encapsulation, crosslinked graft s often act as permanent foreign body materials, similar to GoreTex-based synthetics (30).

It appears that the balance between extracellular matrix deposition and mesh degradation is critical for mesh remodeling and effective tissue reinforcement. Finally, while many investigators reported deposition of “new” matrix at the site of biologic mesh implantations, a typical scar plate developed as a part of normal wound healing could mimic regeneration. Distinguishing regenerated collagen within degraded scaffold versus fibrotic scar formation remains a challenge, even for experienced tissue histopathologists (30).

It is worth mentioning that one of the major limitations of biologic mesh research is the common animal models used. Most investigations are reported in rodents, guinea pigs, or minipigs. Those animals are chosen due to relative ease of implantation, low cost, and possible availability of genetic variants (mice) for future studies. However, tissue responses in those animals probably do not directly compare with those of humans, especially in the long term. Several investigators, on the other hand, have utilized the Old World primates (21) (31). Those animals are highly homologous to humans in key components of the immune system. However, even though primates appear to be the most suitable animal model for translational biologic mesh research, ethical and financial constraints preclude widespread use of the primates for basic mesh investigations. Moreover, the most limiting aspect of animal research is not only in the chosen species but also that most investigations are performed under essentially ideal conditions. Typical patients undergoing biologic mesh repairs are likely to have multiple comorbidities, large abdominal wall defects, obesity, and various degrees of wound contamination. However, essentially no comparative investigations have been performed in anything but healthy animals.

Our experimental study "in vitro"

The laboratory experiment had been performed under the direction of Prof. Paola Campomenosi of the Biotechnologies and Life Science Department in the University of Insubria.
This project aimed to understand aspects of the interaction between different matrices used in abdominal wall repair surgery and host cells, by using *in vitro* cell culture, investigating the molecular processes activated by fibroblasts during their interaction with different types of biological and synthetic matrices, comparing the fibroblast-matrix interactions morphologically, monitoring cell proliferation and the expression of genes involved in the deposition and reabsorption of collagen, as well as some cytokines and markers of differentiation into myofibroblasts.

Pilot experiment

The experimental project was proceeded by a pilot experiment in order to identify the right protocols to measure the different parameters we were interested and obtain reliable data.
During the pilot study the preparation of the meshes for cell culture, the ideal number of cells to seed, the supplement media and the best method for the evaluation of cell growth on different types of matrices, for cell counting, for RNA extraction and for measurement Matrix Metalloproteinases (MMP) were defined.
Preliminary data suggested that more extensive washing step was needed for the Strattice mesh than what was expected.
As supplemented media, Lyset allowed a very efficient growth of primary fibroblasts and was found to offer several advantages over fetal bovine serum, normally used in human primary fibroblasts culture. Lyset is a freeze-dried human platelet lysate, whose composition in growth factors is controlled and it is not xenogenic, contrarily than fetal bovine serum.
SEM (Scanning Electron Microscopy) turned out to be the best method to visualize cells growing on mesh surfaces. Giemsa staining coud be used as an alternative to SEM only with the synthetic matrix.
For cell counting two different methods were compared and seemed to give comparable results: a non-enzymatic method (Cell Titer Glo, Promega) based on quantitation of ATP to quantify viable cells (method 1) and an enzymathic (Accutase) detachment followed by manual cell counting (method 2). A comparable average has been obtained with both the methods.

Material and method

Cell culture
Human dermal fibroblast from healthy adult donors (aged 35-45) were obtained from the "Cell Line and DNA Biobank from Patients Affected by Genetic Diseases – NETWORK OF GENETIC BIOBANKS TELETHON". Cells were used for the experiments between the XI and the XIII passage.
Fibroblasts were grown in RPMI-1640 (CARLO ERBA Reagents, Milan, Italy) supplemented with 10% Fetal Bovine Serum (FBS, CARLO ERBA Reagents, Milan, Italy) and 2 mM L-Glutamine (CARLO ERBA Reagents, Milan, Italy). Cultures were incubated at 37°C in 95% humidity and 5% CO2 atmosphere. At confluence, fibroblasts were detached from culture flasks by treatment with trypsin (CARLO ERBA Reagents, Milan, Italy). An aliquot was thawed on average 2 weeks before each experiment to have sufficient numbers of cells.

Type and preparation of matrices

The following four different types of matrix were used:
• Strattice, noncrosslinked acellularized porcine derma, LifeCell-Acelity, Branchburg, NJ,
• Permacol, crosslinked acellularized porcine derma, Covidien, Mansfield, MA,
• Surgisis-Biodesign, noncrosslinked porcine intestinal submucosa, Cook Medical, Bloomington, Ind,
• Prolene, polypropylene highweight, monofilament, Somerville, NJ.

All prosthesis were purchased from the manufacturing companies.
The matrices were cut into small pieces (1×1 cm): Strattice and Permacol were washed with PBS (Phosphate Buffered Saline) at room temperature for eight hours with gentle shaking, changing the washing every 2 hours, whereas Biodesign and Prolene were washed at room temperature for 25 minutes with gentle shaking, changing the washing every 5 minutes. Then each piece of matrix was placed in a well of 12 multiwell tissue culture plates containing complete medium and incubated for 12 hours at 37°C in 95% humidity and 5% CO2 atmosphere.

Experimental setup

Twelve hours after mesh preparation, 50.000 fibroblasts were seeded on the mesh surface in each well and incubated at 37°C in 95% humidity and 5% CO2 atmosphere. Two days after seeding, the matrices were transferred to a new tissue culture plate and fibroblasts were let to grow for 10, 20 and 30 days, before processing samples for analyses.
Three days before the end of treatment, the growth medium was replaced with RPMI-1640 supplemented with 2 mM L-Glutamine and 5% Lyset Kit (Sclavo Diagnostic International by CARLO ERBA Reagents, Milan, Italy), using a 1:4 ratio between the PL:AD reagents.
Two random pieces of matrix were used for each type of mesh for each time point: the first was used for RNA extraction and the second was further subdivided into 4 pieces to be analyzed with SEM (two pieces) and for cell counting (two pieces).
At the end of treatments, the supernatants were collected and stored at -80°C for ELISA experiments.
As a control condition, fibroblasts grown on plastic with RPMI-1640 Medium supplemented with 5% Lyset Kit and 2 mM L-Glutamine were used.

Scanning electron microscopy (SEM)

To obtain 3D imaging by scanning electron microscopy (SEM), samples were fixed with Karnovsky fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate Buffer (pH7.2)) for 1 h at room temperature. Specimens, washed in 0.1 M cacodylate buffer (pH 7.2), were postfixed in a solution of 1% osmium tetroxide and potassium ferrocyanide for 2 h. Each specimen was washed in PBS (pH 7.2) and then immersed in 0.1% osmium tetroxide in PBS for 1 h. Samples were dehydrated in an increasing series of ethanol (70%, 90%, 100%), and subjected to critical point drying with hexamethyldisilazane. Dried samples were mounted on stubs, gold coated with a Sputter K250 coater (Emitech), and then observed with a SEM-FEG XL-30 microscope (Philips, Eindhoven, The Netherlands).

Cell counting

To determine the number of cells grown on the different scaffolds, two methods were used: the first was based on ATP quantization by the CellTiter-Glo kit (Promega, Milan, Italy), whereas the second consisted in enzymatic detachment of cells from the matrices, followed by manual cell counting. Enzymatic detachment was performed by incubating matrices with Collagenase from Clostridium Histolyticum, Type IA (Sigma-Aldrich, Milan, Italy) at a final concentration of 5 mg/ml directly diluted in Accutase solution (CARLO ERBA Reagents, Milan, Italy), for 25 minutes at 37°C. Then 100 µl of Trypsin were added for 5 minutes at 37°C.

RNA Extraction and qPCR Analysis

Fibroblasts grown on the matrices were manually scraped in 300 µl of TriReagent (Sigmaaldrich, Milan, Italy) on ice, then lysates were collected in tubes and stored at -80°C. Total RNA was extracted using a Direct-zol RNA MiniPrep (ZYMO RESEARCH, EuroClone, Milan, Italy) following manufacturer instructions. RNA samples were quantified with a NanoDrop 2000c (ThermoFisher, Life Technologies Italia, Milan, Italy) and run on an agarose gel. For real-time quantitative PCR (qPCR), cDNA was obtained from 750 ng of RNA by using the iScript cDNA synthesis kit (Biorad, Milan, Italy). Gene expression analysis was performed in triplicate using a CFX96 thermal cycler (Biorad, Milan, Italy) and the iTAQ Universal Sybr Green Supermix (Biorad, Milan, Italy). Relative mRNA quantification was obtained by applying the 2^-DDCq method 14, using the geometric average of the Cqs of two reference genes, namely beta-2-Microglobulin and GAPDH, for normalization purposes. Melting curve analysis was performed to ensure that single amplicons were obtained for each target.
Primers for the genes under investigation were designed to have at least one of the primers in the pair designed on an exon-exon junction, or to encompass at least one intron. For primer design and thermodynamic analysis of their quality the following programs were used: the Primer-Blast tool at NCBI(http://www.ncbi.nlm.nih.gov/tools/primerblast/),OligoCalc(http://biotools.nubic.northwestern.edu/OligoCalc.html) and the IDT SciTools (http://eu.idtdna.com/pages/scitools). Primer sequences are reported in Table 2: the selected genes are specific for the types of collagen present in the matrices and are those mainly involved in the extracellular matrix metabolism.

Gene	Ref_Seq		Sequences (5' --> 3')	Product length
MMP1	NM_001145938.1; NM_002421.3	Primer Fw Primer Rv	AAGGTCTCTGAGGGTCAAGCA TCCCGATGATCTCCCCTGAC	59
MMP2	NM_001127891.2; NM_001302508.1; NM_001302509.1; NM_001302510.1; NM_004530.5	Primer Fw Primer Rv	GCCAAGTGGTCCGTGTGAA GCTGTTGTACTCCTTGCCATTG	86
MMP9	NM_004994.2	Primer Fw Primer Rv	TTCTGCCCGGACCAAGGATA TCCGGCACTGAGGAATGATCT	89
TIMP1	NM_003254.2	Primer Fw Primer Rv	GCAATTCCGACCTCGTCATCA GTCAGCGGCATCCCCTAAG	134
TIMP2	NM-003255.4	Primer Fw Primer Rv	GCTGCGAGTGCAAGATCAC GGTGCCCGTTGATGTTCTTC	108
MMP13	NM_002427.3	Primer Fw Primer Rv	GGAATTAAGGAGCATGGCGAC GCCCAGGAGGAAAAGCATGA	76
COL1A1	NM_000088.3	Primer Fw Primer Rv	CAAGACGAAGACATCCCACCAA ACGTCATCGCACAACACCTT	128
COL1A2	NM_000089.3	Primer Fw Primer Rv	TGAAGATGGTCACCCTGGAAAA CACCCTGTGGTCCAACAACT	65
COL3A1	NM_000090.3	Primer Fw Primer Rv	GGTCCAACTTCACCCTTAGCA TGCACCGCTTCACCCTTAGCA	90
CTGF	NM_001901.2	Primer Fw Primer Rv	TGCACCGCCAAAGATGGT GCAGACGAACGTCCATGCT	148
IL6	NM_000600.4; NM_001318095.1	Primer Fw Primer Rv	TAGTGAGGAACAAGCCAGAGC TTGGGTCAGGGGTGGTTATTG	104
ACTA2	NM_001141945.1; NM_001613.2	Primer Fw Primer Rv	GGCAAGTGATCACCATCGGA GTGGTTTCATGGATGCCAGC	100
GAPDH	NM_001289746.1; NM_001289745.1; NM_002046.5	Primer Fw Primer Rv	GAAGGTGAAGGTCGGAGTC GAAGATGGTGATGGGATTTC	226
B2M	NM_004048.2	Primer Fw Primer Rv	AGGCTATCCAGCGTACTCCA ATGGATGAAACCCAGACACA	102

Table 2. Sequences of primers used for gene expression analysis in this study.

Quantification of secreted proteins by ELISA

The concentration of human IL-6, MMP1 and MMP2 in cell culture supernatants from fibroblasts grown on the different types of matrices was measured using a Human IL-6 (Thermo Scientific, Life Technologies Italia, Milan, Italy), Human MMP1 (Thermo Scientific, Life Technologies Italia, Milan, Italy) and Human MMP2 (NOVEX, Life Technologies Italia, Milan, Italy) colorimetric ELISA, according to manufacturer's instructions.

Statistical Analysis

Data from analysis of gene expression and from quantification of secreted proteins from three independent experiments were compared using analysis of variance (ANOVA) with post hoc Duncan's test by Statistica software program 7 version.
Cell numbers counted on the different matrices and ELISA results were compared using analysis of variance (ANOVA) by GraphPad Prism statistical software program 4.02 version.

Results

Morphologic analyses by SEM

Morphological examination by scanning electron microscopy (SEM) of fibroblasts grown on the different types of meshes at 10, 20 and 30 days after cell seeding on the different matrices was performed.
Examination and acquisition of SEM images were performed for all the four meshes inserted in the study, in collaboration with Prof. Annalisa Grimaldi and Prof. Terenzio Congiu in the SEM facility of University of Insubria.

Strattice mesh (fig. 1) showed an increasing number with time of adherent cells on the matrix surface (upper surface, where cells were seeded), indicating that they are healthy and proliferating. Cells were present also in the bottom surface, although at lower numbers compared to the upper side. Cell distribution was uniform and cells were strongly interacting with the matrix. Morphologically, production of new extracellular matrix seemed occur.

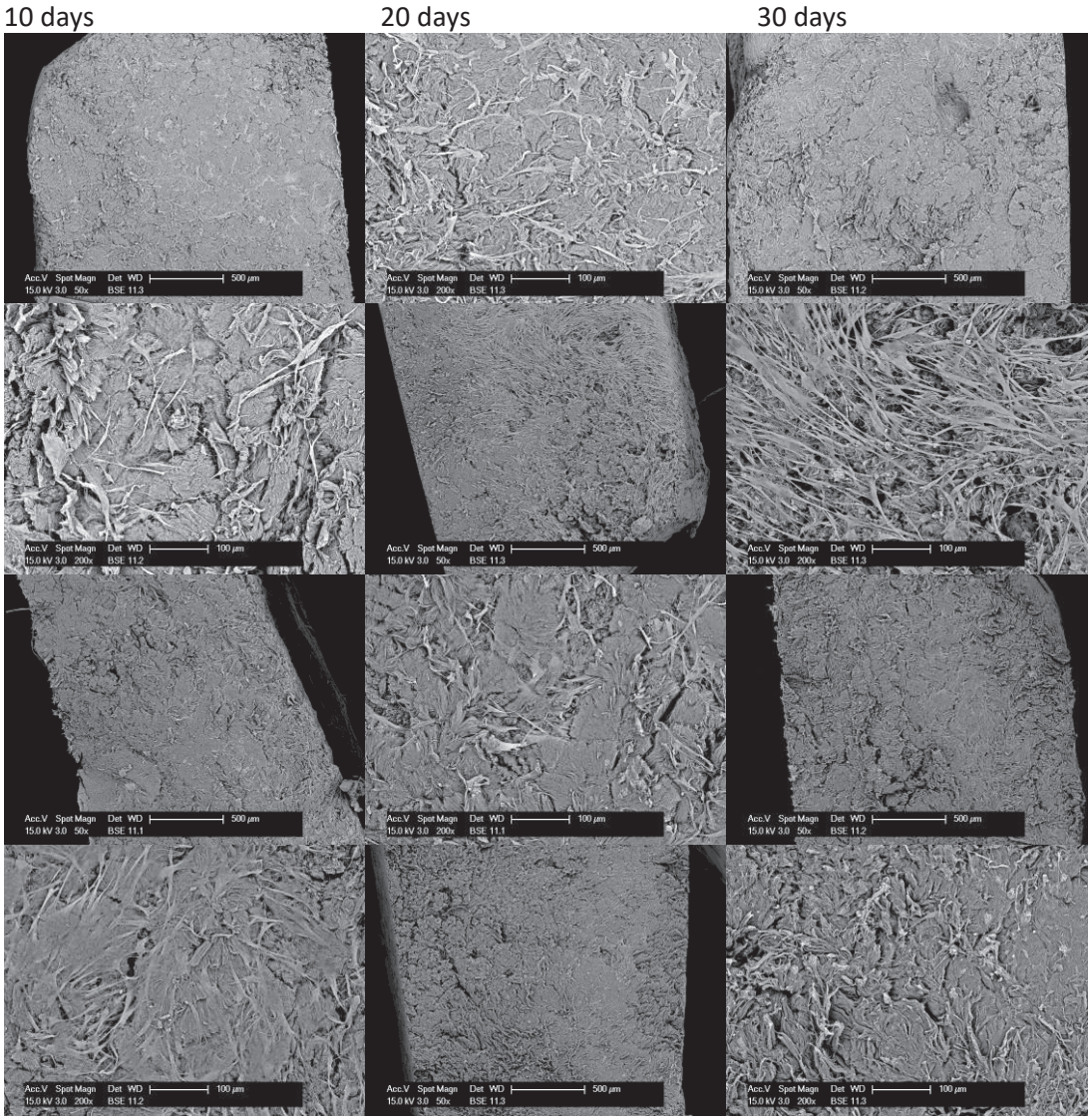


Fig. 1 Strattice mesh at SEM analyses at 10, 20 and 30 days. At each time both upper and bottom side are reported at 50x and 200x magnification

In Pemacol matrix at 10 days after seeding, cells were distributed unequally on the surface. The 50x magnification picture (fig. 2) shows a part of the matrix colonized by cells, while another part is free of cells. The same unequally distribution was observed in the replicate sample (fig.3), as a proof that experiment was led in a correct way. Unexpectedly, at 20 days the number of cells on the surface was lower than that at 10 days, whereas at 30 days the sample showed a monolayer of cells. Very few cells were found on the bottom of the matrix (opposite side respect to seeding).

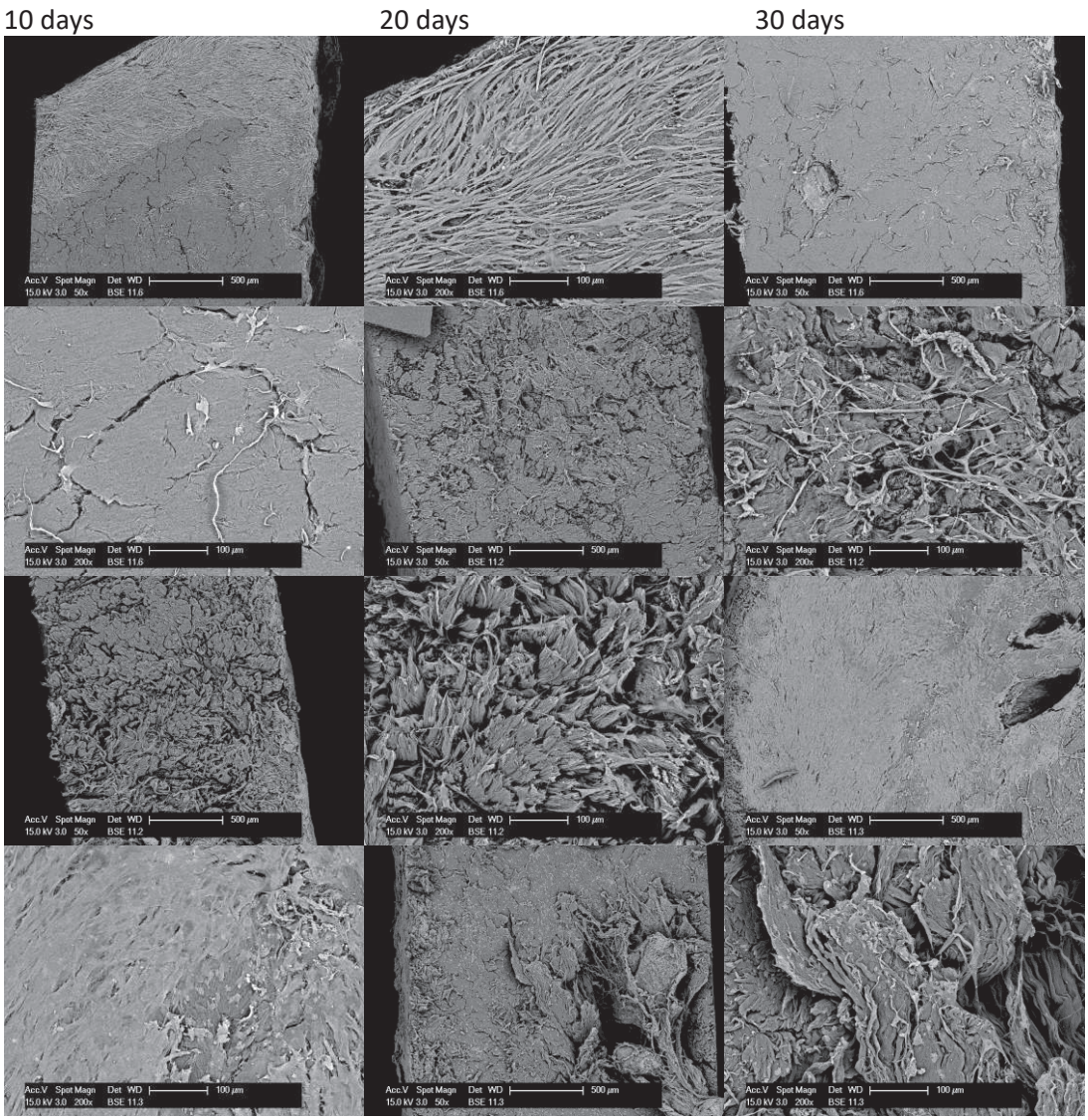


Fig. 2 Permacol mesh at SEM analyses at 10, 20 and 30 days. At each time both upper and bottom side are reported at 50x and 200x magnification

10 days 20 days

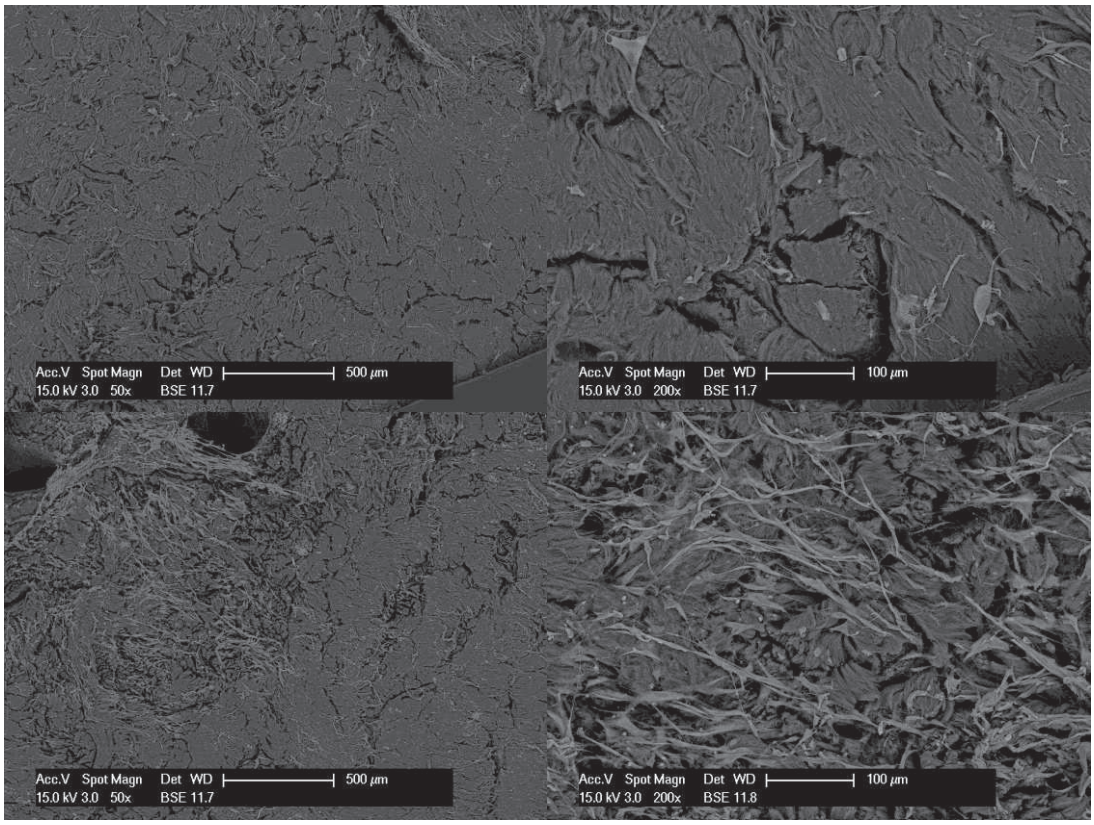


Fig. 3 The replicate sample of Permacol showed the same non homogeneous distribution of cells. Only the upper side is showed at 10 and 20 day, at 50x and 200x magnification

The Surgisis-Biodesign matrix (fig. 4) presented the largest number of cells, in particular at early times. At 10 days cells covered the surface of the matrix and some cells were found also on the bottom side of it. At 20 days cells began to peel off. They were arranged in different layers, but did not appear to really penetrate the matrix layers. At 30 days a fewer number of cells were observed.

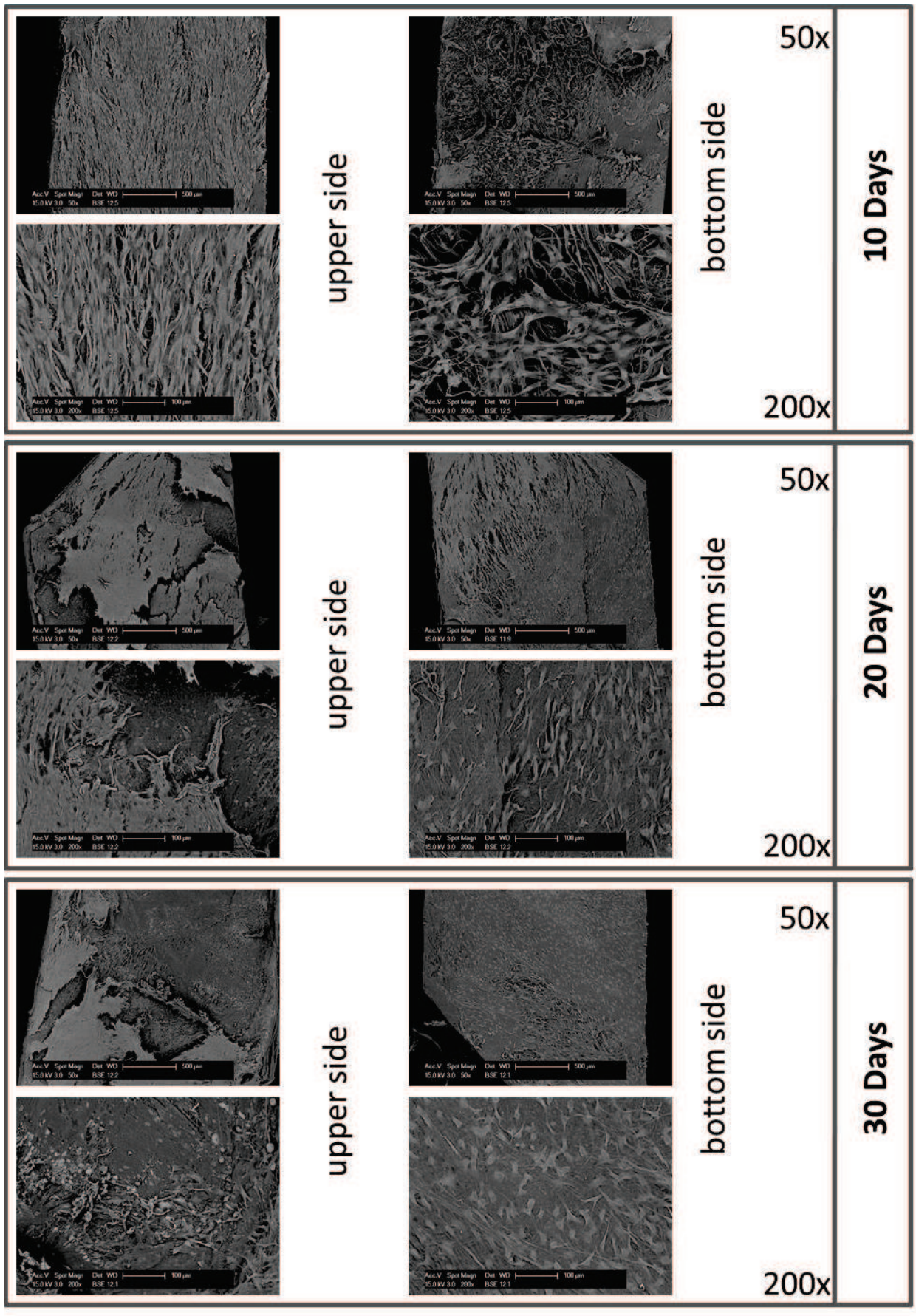


Fig. 4 Biodesign mesh at SEM analyses at 10, 20 and 30 days. At each time both upper and bottom side are reported at 50x and 200x magnification

Very few cells growing on Prolene mesh were observed at any times.
Cells did not appear to attach and grow easily on this material.

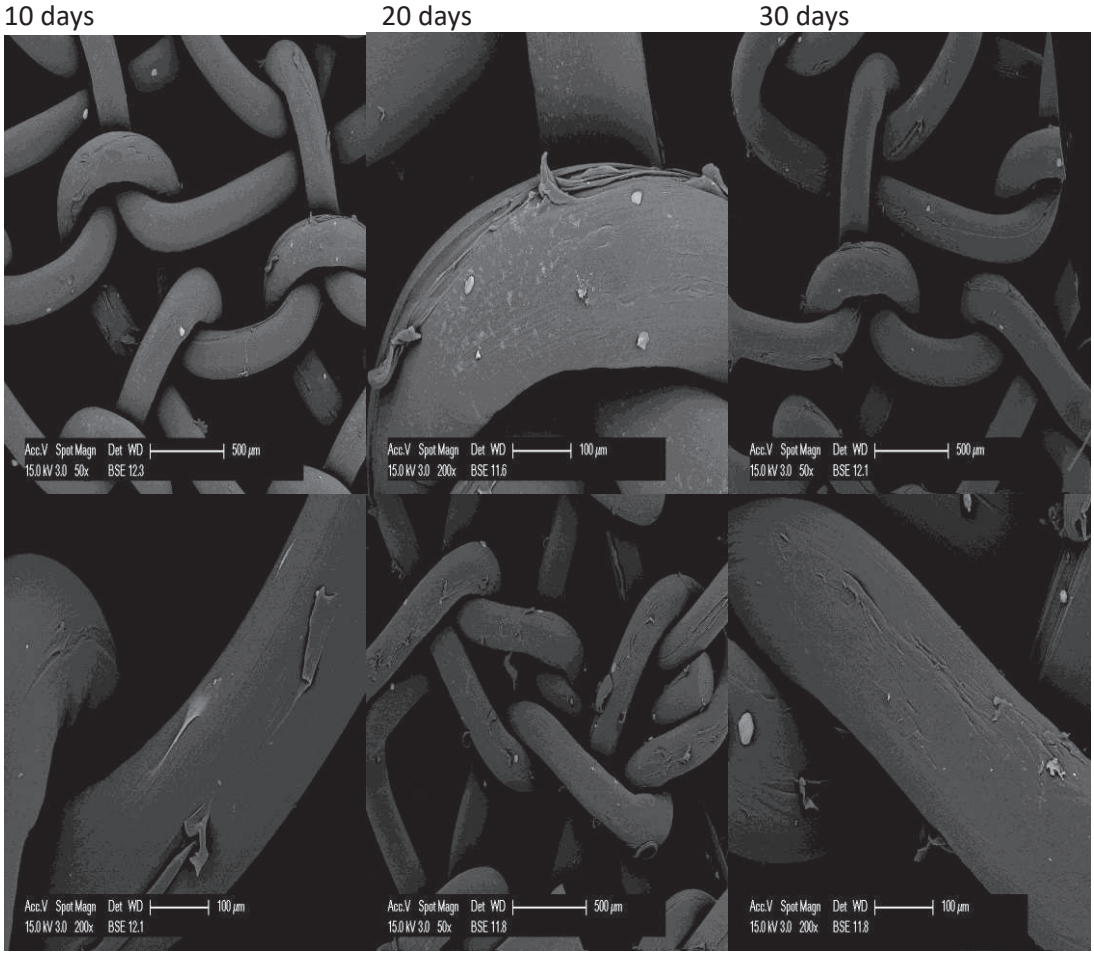


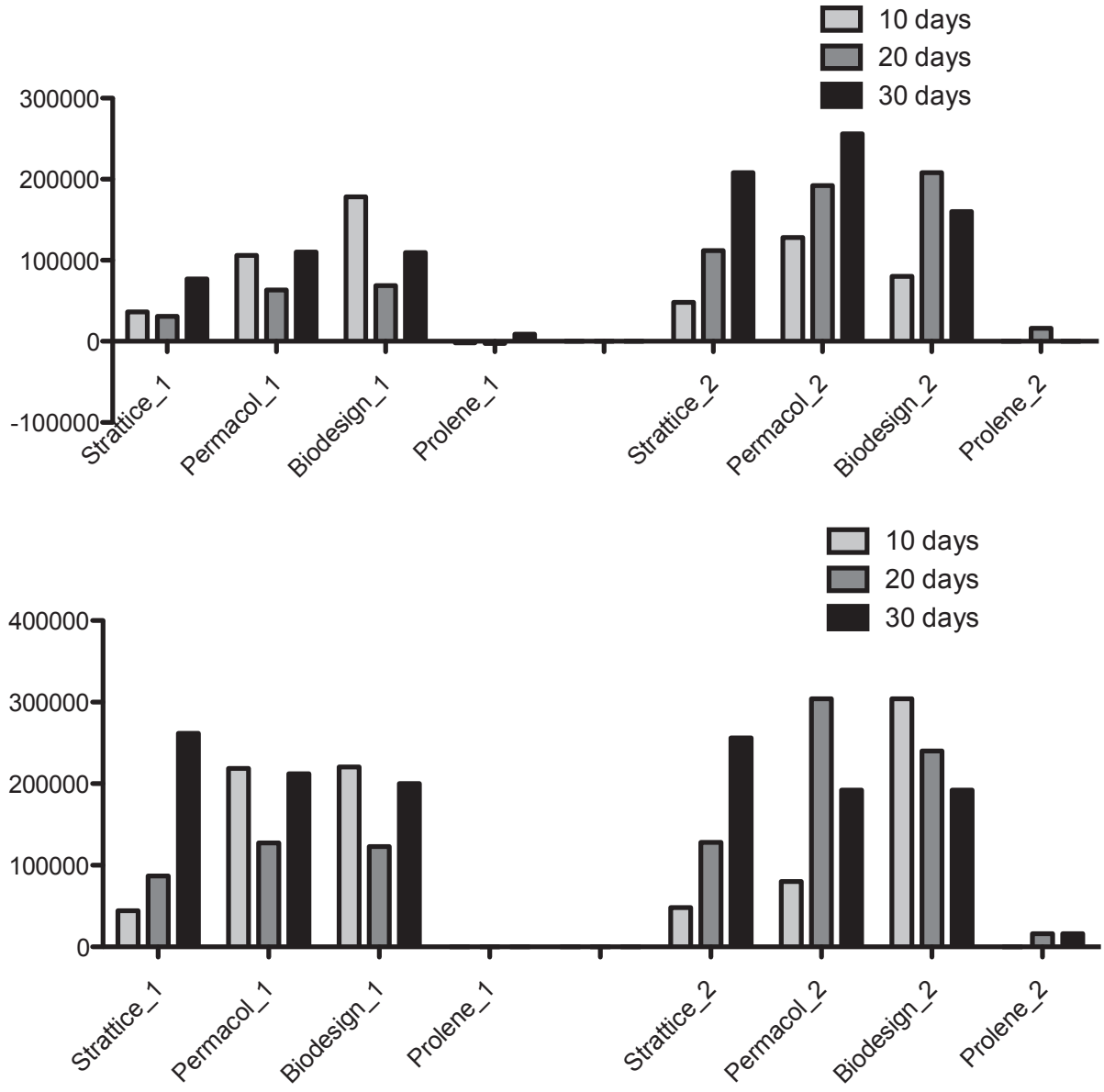
Fig. 5 Prolene mesh at SEM analyses at 10, 20 and 30 days. At each time upper side is reported at 50x and 200x magnification

Cell counting and growth curves

On Strattice mesh a lower number of cells was counted at 10 days and 20 days than number present on other biological matrixes, but it increased steadily time by time.

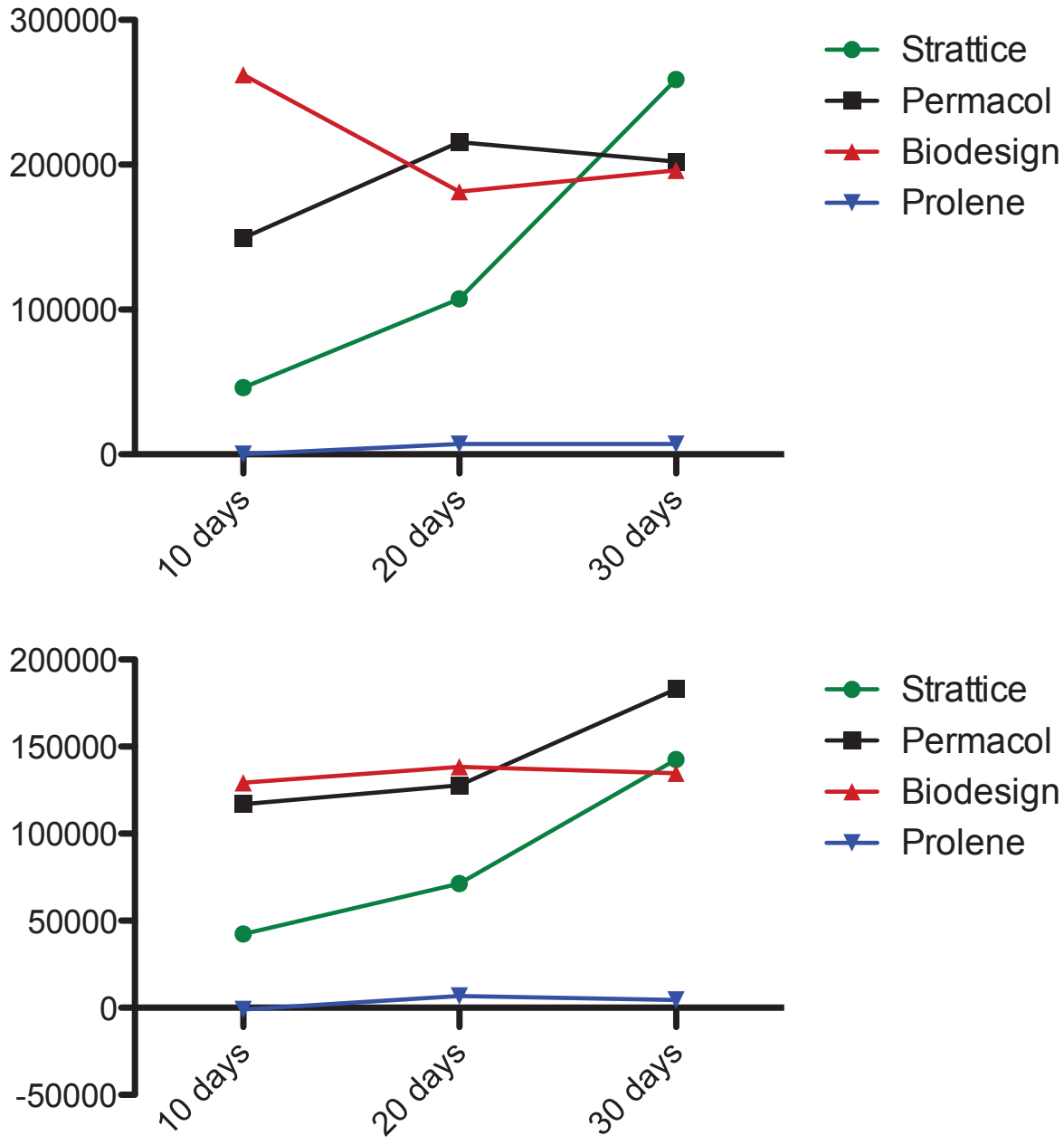
The number of cells growing on the Permacol matrix at each time point seemed to be quite variable, depending on the mesh piece examined.

The cells counted on Surgisis-Biodesign at 10 days is quite always the highest counted in all meshes, but unexpectedly the number was reduced in the following time point.



Graph 1 Cell growth at 10, 20 and 30 day on the four different meshes. The count has been performed with method 1 and 2. The experiment has been accomplished in November 2015 and repeated in February 2016

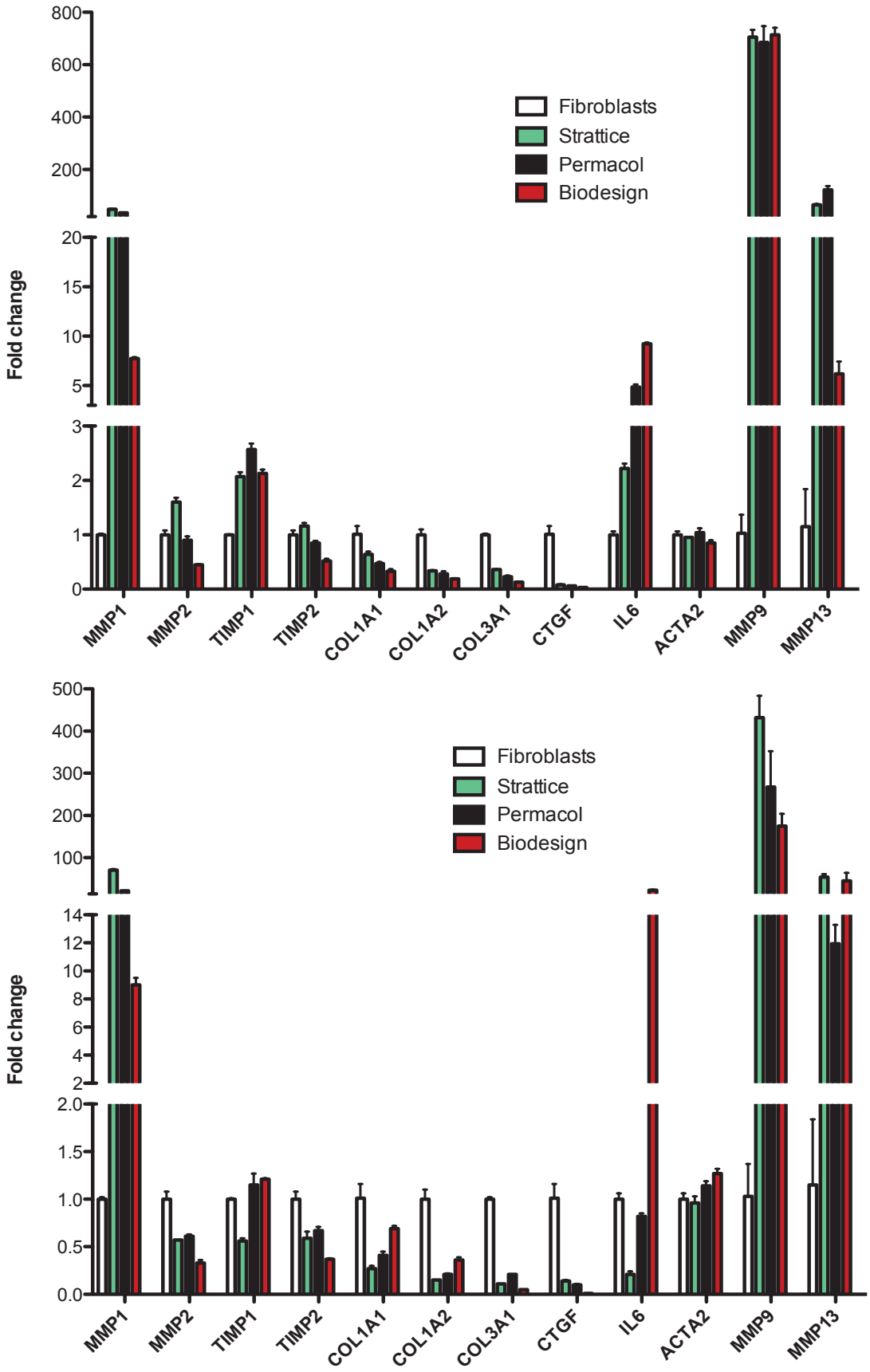
In the Graph 2 the average of number of cells, counted with the two different methods, is reported for each matrix at each time point, for both the experiments (November 2015 and February 2016) to give an idea of the cell growth kinetics on each matrix.
Cell counting on Strattice mesh was always increasing by time and in November 2015 the counting at 30 days reported a cell number superior than on the other meshes.
On Permacol mesh, cell counting at 20 and 30 days was just a little highest than number present al 10 days.
On Surgisis-Biodesign mesh, cell growth evolution by time was decreasing in November 2015 and stable in February 2016.



Graph 2 Cell growth for each meshes at each time point in the two experiment (November 2015 and February 2016)

Gene expression analyses

The graph 3 shows the expression of specific genes from primary fibroblasts grown for 20 (upper panel) and 30 (lower panel) days in the different types of mesh. The low amount of RNA extracted at 10 days of incubation did not allowed analyses of the whole set of genes.
It was not possible to complete RNA extraction from the very few cells grown on Prolene mesh.
MMP1 and MMP13 metalloprotease show a similar pattern of expression, although the latter is less abundant in absolute terms (from the cycle threshold).
In general, MMPs increased in fibroblasts grown on matrices containing collagen compared to those grown on plastics (white bar).
Cells grown on Strattice and Permacol showed a similar behaviour, strongly inducing all MMPs tested (MMP1, MMP2, MMP9, MMP13), while fibroblasts grown on the Biodesign matrix expressed lower levels of MMPs.
IL6 instead showed maximal expression in cells grown on Biodesign, followed by cells grown on Permacol and Strattice at both time points.
No new deposition of collagen did not occur at the time points analyzed. On the contrary, a reduction in their expression was reported, compared to fibroblasts grown on plastic.



Graph 3 Expression of the genes at 20 days (upper panel) and at 30 days (lower panel)

Expression of collagen genes

First, we decided to investigate if the first transcriptional program activated in fibroblasts grown on the different types of mesh indicated new matrix deposition or degradation of the scaffold. As primary dermal fibroblasts were used in this study, we aimed to analyze the production of collagen I and collagen III. Fibroblasts grown for 10 days on all biological scaffolds showed a significant decrease in COL1A1 and COL3A1 transcripts compared to fibroblasts grown on plastics ($p<0.005$, ANOVA with posthoc Duncan's test) (Figure 6, left panel). At 30 days a reduction in transcript levels for cells grown on all matrices compared to control cells was still observed, albeit less pronounced than at 10 days (Figure 6, right panel). No significant difference among the different biological matrices was observed.
The same reduction was observed for COL1A2 transcript at 10 days of culture (Figure 6, left panel). The reduction was still significant at 30 days for fibroblasts grown on Strattice and Biodesign matrices (Figure 6, right panel), but not for cells grown on Permacol.
We concluded that new extracellular matrix deposition does not occur in the selected time frame.

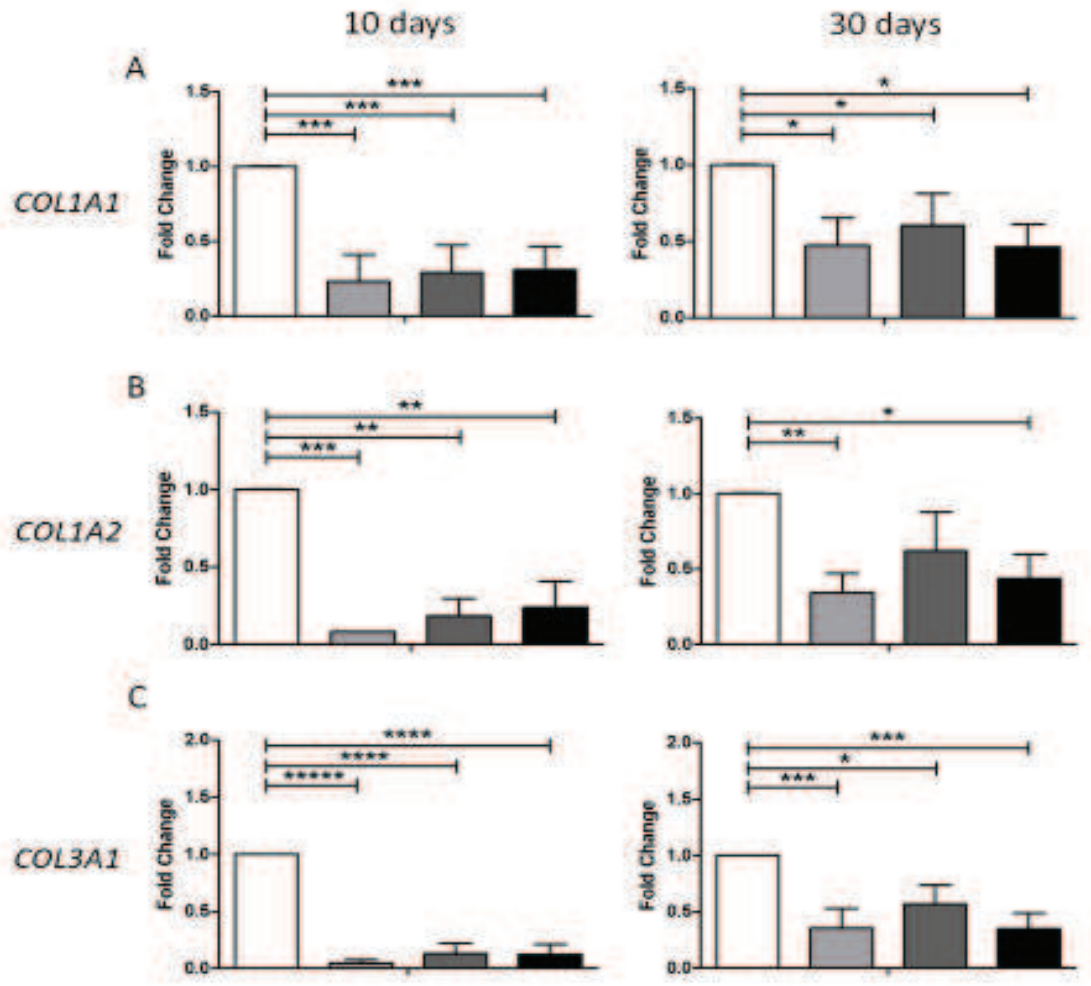


*Figure 6. Changes in the levels of collagen transcripts following growth on Strattice, Permacol and Biodesign matrices for 10 or 30 days compared to control condition. A. COL1A1; B. COL1A2; C. COL3A1. White bar: expression from fibroblasts grown on plastics (control); light grey bar: expression from fibroblasts grown on Strattice; dark grey bar: expression from fibroblasts grown on Permacol; black bar: expression from fibroblasts grown on Biodesign. Values are means of four independent experiments ± SE. The results were analyzed by ANOVA. * indicates significant values (* $p < 0.05$; ** $p < 0,01$; *** $p < 0,005$; **** $p < 0,001$; ***** $p < 0,0005$).*

Expression of extracellular matrix degrading enzymes

We therefore investigated if the predominant process in this early timeframe was extracellular matrix degradation, by measuring the expression levels of metalloproteinases.
MMP-1 showed on average an increase in transcript levels in fibroblasts grown on the different biological matrices compared to cells grown on plastics (Figure 7, left panel).
The increase was not significant at 10 days, due to the high variability in the biological replicates; at later times (20 days, data not shown and 30 days, Figure 7, right panel), cells grown on Strattice had the highest expression of MMP-1, and the difference was significant not only with respect to control cells, but also to fibroblasts grown on the other two biological meshes (Figure 7). Fibroblasts grown on the other scaffolds still showed a significant 5-10 fold increase compared to control cells at 20 (data not shown) and 30 days (Figure 7).
The data on MMP-1 transcript were confirmed by measurement of MMP-1 protein in cell supernatants by ELISA. We found the highest levels of this metalloproteinase in cells grown on Strattice at both 10 and 30 days, whereas cells grown on Biodesign showed the lowest increase compared to control cells, among the three meshes, at both times (Figure 7). MMP-2 transcript levels were slightly increased in cells grown on Strattice compared to control cells at all times, whereas in the Biodesign matrix we found a reduction of expression by 50% compared to control cells at both times (Figure 7). The expression was more variable when cells grown on Permacol were analyzed (Figure 7).
MMP-2 transcript level significantly differed not only from control condition (Strattice vs control, Biodesign vs control) but also among the various biological matrices at 30 days. MMP-2 protein levels were higher in the Strattice samples than in controls at 30 days, confirming the data obtained with transcript analysis. In the Biodesign sample no change in MMP-2 protein was observed at 10 days compared to control cells, but the protein was expressed 8 fold compared to control cells at 30 days in this matrix, in spite of lack of detectable increase in transcript (Figure 7). However, except for Strattice, the differences described in MMP-2 protein were not statistically significant due to high variability among the three replicates.
Regarding MMP-9 transcript levels, the increase we observed with all biological matrices we could analyze was not significant, due to high variability among experiments.
Moreover, for cells grown on Strattice for 10 days, the number of cells did not allow to extract sufficient amounts of RNA to analyze all genes of interest (Figure 7). Also for MMP13 we did not observe any significant changes in any of the conditions tested (Figure 7).

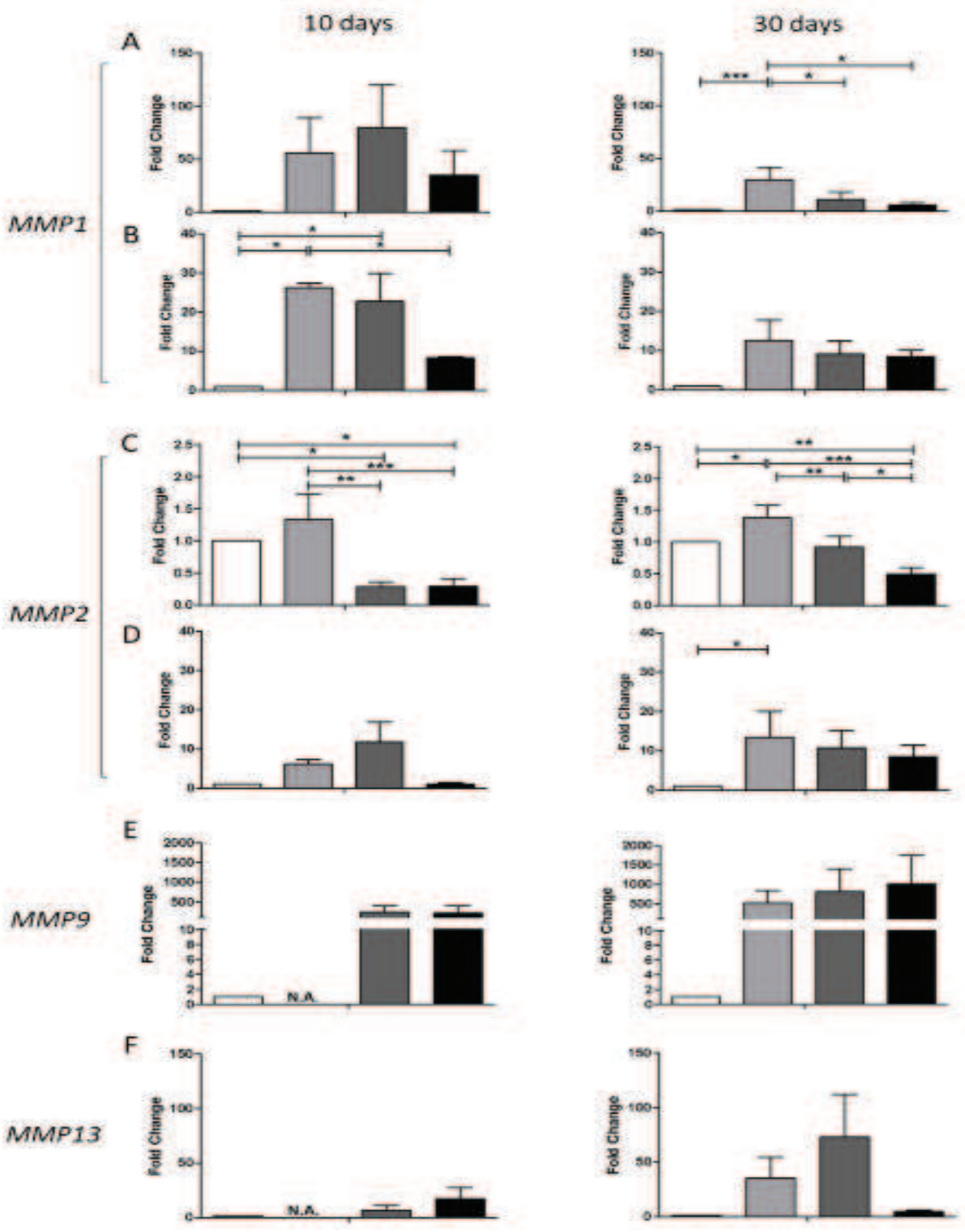


*Figure 7. Changes in the levels of metalloproteinases transcripts and proteins following growth on Strattice, Permacol and Biodesign matrices for 10 or 30 days, compared to control condition. A. MMP1 transcript and B. protein; C. MMP2 transcript and D. protein; E. MMP9 transcript; F. MMP13 transcript. White bar: expression from fibroblasts grown on plastics (control); light grey bar: expression from fibroblasts grown on Strattice; dark grey bar: expression from fibroblasts grown on Permacol; black bar: expression from fibroblasts grown on Biodesign. Values are means of four independent experiments ± SE. The results were analyzed by ANOVA. * indicates significant values (* $p < 0.05$; ** $p < 0,01$; *** $p < 0,005$; **** $p < 0,001$; ***** $p < 0,0005$). N.A. indicates that the sample was not analyzed since the amount of RNA extracted from the sample was not sufficient to analyze all genes of interest*

Expression of metalloproteinase inhibitors

Given the high levels of MMP-1 expressed by fibroblasts grown on biomatrices, we asked if its specific inhibitor, TIMP-1 was also already expressed. Although a slight increase in TIMP-1, but not TIMP-2, analyzed as control, was observed at 30 days after cell seeding in all biological matrices, it did not reach statistical significance (Figure 8A and B, respectively).

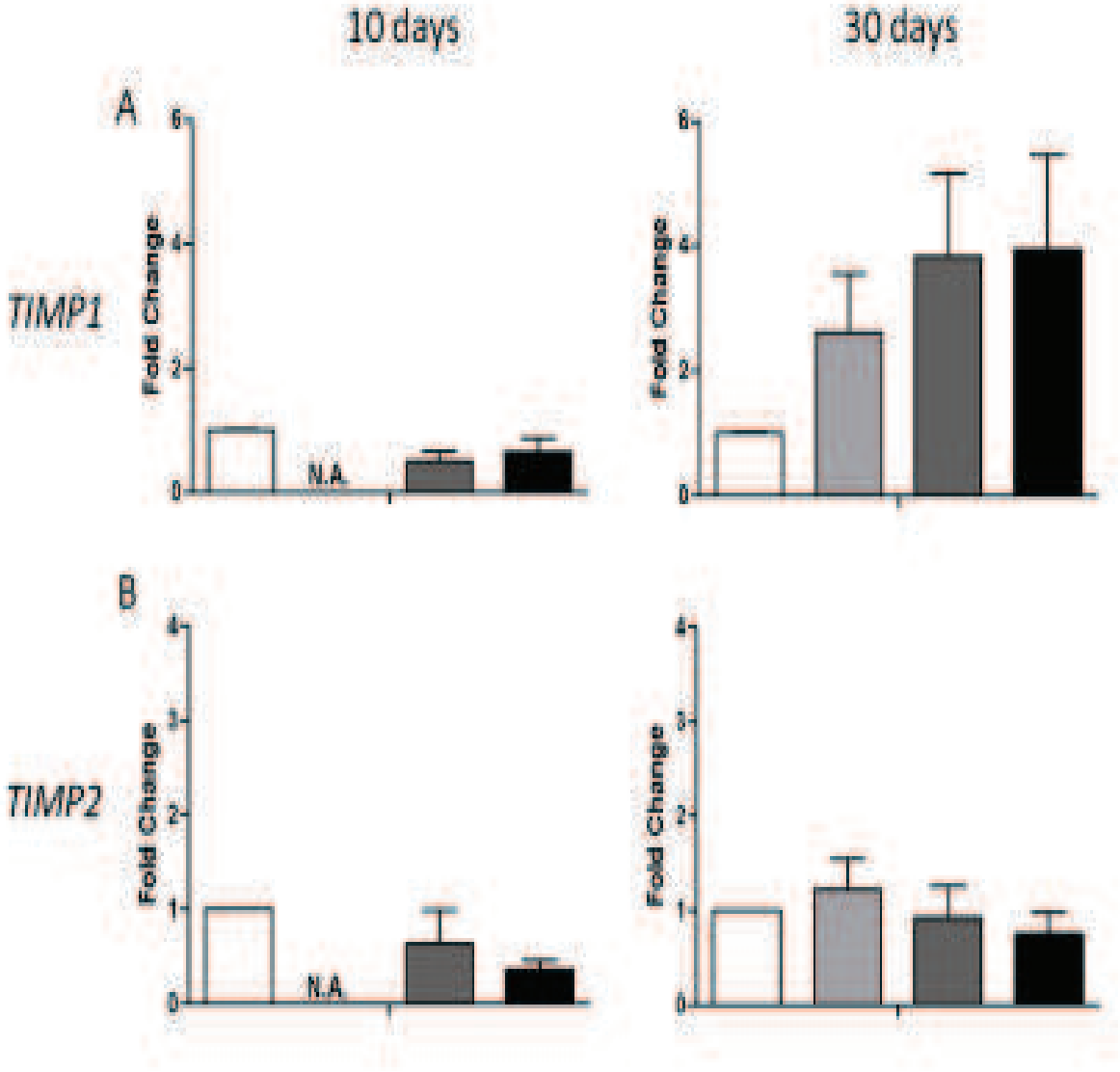


*Figure 8. Changes in the levels of metalloproteinase inhibitors' transcripts following growth on Strattice, Permacol and Biodesign matrices for 10 or 30 days, compared to control condition. A. TIMP1 transcript; B. TIMP2 transcript. White bar: expression from fibroblasts grown on plastics; light grey bar: expression from fibroblasts grown on Strattice; dark grey bar: expression from fibroblasts grown on Permacol; black bar: expression from fibroblasts grown on Biodesign.Values are means of four independent experiments ± SE. The results were analyzed by ANOVA. * indicates significant values (* p < 0.05; ** p < 0,01; *** p < 0,005; **** p < 0,001; ***** p < 0,0005).*

Expression of Cytokines

We also investigated if specific cytokines, namely Interleukin-6 (IL-6) and Connective Tissue Growth Factor (CTGF), were differentially expressed by fibroblasts grown on the different types of mesh. Interleukin-6 (IL-6) transcript showed a slight decrease or no change in fibroblasts grown on the different biological matrices at 10 days. Expression was increased at 30 days but only cells grown on Biodesign expressed significantly different levels compared to the control as well as to the other biological matrices (Figure 9A). IL-6 protein levels, measured by ELISA, were not affected by any of the matrices at 10 days, but appeared to be increased, albeit not significantly, in supernatants from cells grown on all biological meshes compared to control cells at 30 days (Figure 9A, right panel).

CTGF, together with alpha-smooth muscle actin (α-SMA), product of the ACTA2 gene, are considered markers of myofibroblast differentiation and were both examined to investigate the possible differentiation of fibroblasts into myofibroblasts, responsible for induction of fibrotic reaction, when cultured on the different scaffolds.

A highly significant reduction in CTGF transcript was observed for fibroblasts grown on all types of biological meshes examined already at 10 days, compared to cells grown on plastics (Figure 9 B). The reduction was stable for the time period of our experiments, as it was observed also at 30 days (Figure 9B). Expression of Alpha-smooth muscle actin (α -SMA) α-SMA transcript levels were reduced in fibroblasts grown on biological meshes compared to cells grown on plastics at both 10 and 30 days (Figure 9C).

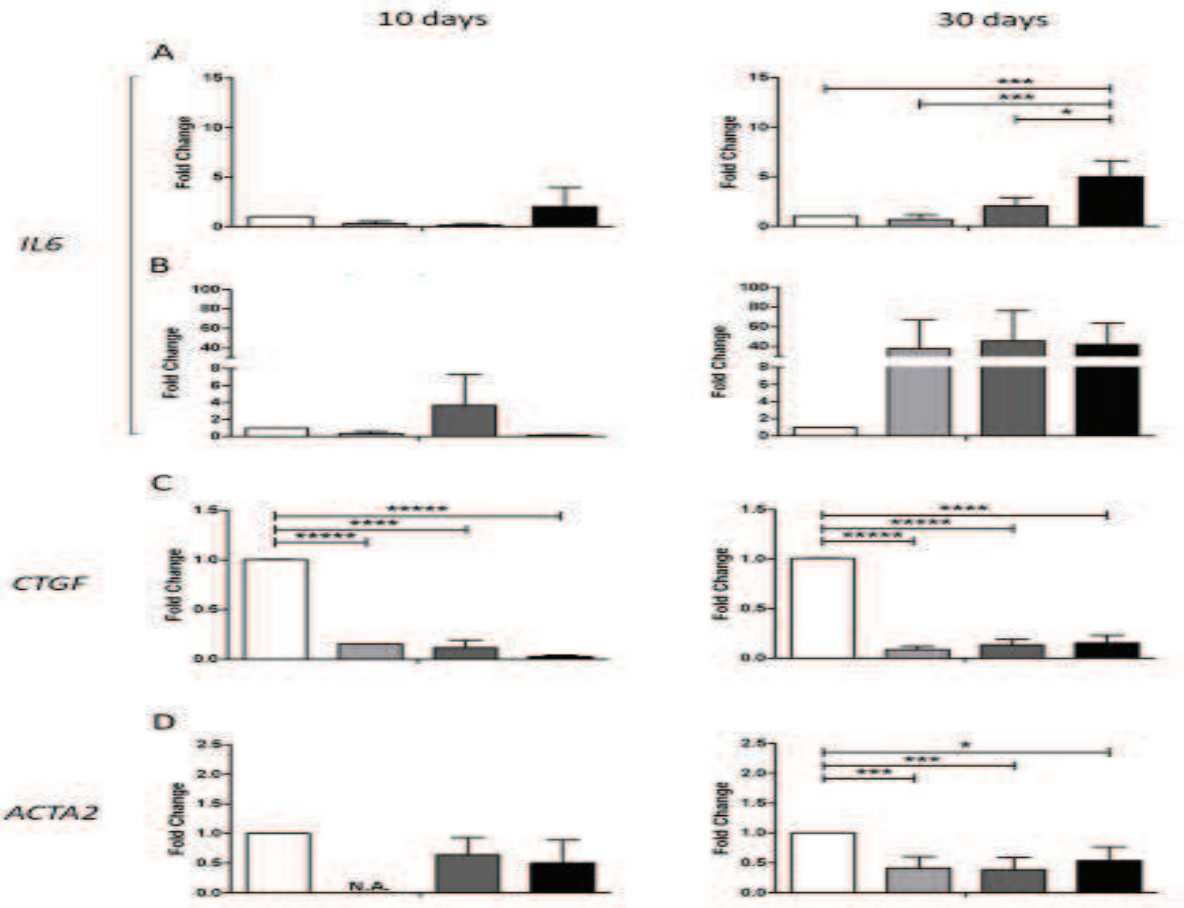


Figure 9. Changes in the levels of cytokines and -smooth muscle actin transcripts and proteins following growth on Strattice, Permacol and Biodesign matrices for 10 or 30 days compared to control condition. A. IL6 transcript and B. protein; C. CTGF transcript; D. ACTA2 transcript. White bar: expression from fibroblasts grown on plastics; light grey bar: expression from

*fibroblasts grown on Strattice; dark grey bar: expression from fibroblasts grown on Permacol; black bar: expression from fibroblasts grown on Biodesign. Values are means of four independent experiments ± SE. The results were analyzed by ANOVA. * indicates significant values (* $p < 0.05$; ** $p < 0,01$; *** $p < 0,005$; **** $p < 0,001$; ***** $p < 0,0005$).*

Discussion

In this work, we aimed to investigate the interaction of human primary fibroblasts from derma of healthy adult individuals with biological or synthetic scaffolds in vitro, by means of cell counting, SEM and gene expression analyses. Three types of biological mesh for hernia repair, differing among them for tissue of origin and/or presence of crosslinking were studied, together with a synthetic mesh. The physicochemical properties of these materials have been described previously in an elegant work by Deeken et al. (24).

In our study, we noticed that the time range of 30 days has been maybe too short for demonstrating significant differences among the examined meshes: the cell growth on the biological grafts at 30 days is quite comparable, while on Prolene it seems to be very poor. Moreover, cells seeded at 10, 20 and 30 days would tend not to produce new collagen, but initially seem to promote the degradation of the matrix above all. Gene expression indicates that within 30 days there is mainly activation of metalloproteases, while the synthesis of collagen would seem a later event.

Fibroblasts showed an initial difficulty to attach and grow on Strattice: in preliminary experiments, extensive washing of this material was found to be essential for cells to attach. After attachment, cells firmly adhered to Strattice and proliferated, as confirmed by the number of cells retrieved during later time points. The behaviour of cells on Permacol scaffolds was similar to Strattice, except cells attached to this material more easily, as shown by the higher number of cells recovered at 10 days compared to Strattice.

These materials have the same origin, porcine derma, but Permacol is also cross-linked.

Therefore, it is possible that preparation of the matrices before commercialization yields scaffolds that are more or less suitable for cell attachment. On the Biodesign samples, the number of cells was initially higher than those on the other biological matrices but also showed greater variability, as demonstrated by both SEM analyses and inspection of growth curves.

Taking into account also the cell numbers obtained after cell counting, we hypothesized that some cells, loosely interacting with the matrix, detached from the surface and were lost during processing of Biodesign samples. Alternatively, it may also be that cells efficiently proliferated to confluence on this matrix and could not grow further.

Indeed, at 30 days the number of cells retrieved from all biological matrices was comparable. This might represent the highest cell density that can be grown on the surface under examination (1 cm2). However, except for Biodesign, we did not observe cell confluence on the matrices during SEM analyses. It is possible that cell adhesion on derma scaffolds is influenced by the direction of collagen fibers. This was shown to be the case for cells grown on membranes for tendon repair (16).

The discrepancy between the number of cells observed by SEM analysis and that obtained by cell count on the Biodesign scaffold could be due to weak interaction of cells with the scaffold or to the possible exfoliation of the scaffold during immersion in medium for the relatively long periods used in the work.

In a work aimed at coating various meshes with fibroblasts or mesenchymal stem cells (MSC) in vitro, human fibroblasts bound to Strattice matrix less efficiently than mouse fibroblasts or MSC (17). In the same work, fibroblasts failed to coat Marlex, a heavyweight, monofilament, polypropylene mesh, confirming the results we obtained with a similar material, Prolene (17). Also in vivo Strattice showed poor incorporation, but no adhesions formation.

In this study, we analyzed expression of several genes involved in extracellular matrix metabolism. We found a significant reduction in the expression of COL1A1, COL1A2 genes (components of collagen I) as well as of COL3A1 (collagen III) in fibroblasts grown on the different biological

matrices compared to fibroblasts grown on plastics, suggesting that no new deposition of collagen occurred in the investigated time frame. However, expression may start at later times, as at 30 days the reduction in expression was less marked than at 10 days. Analyses at longer times would be required to address this question.
We also evaluated the expression of matrix metalloproteinases produced by dermal fibroblasts, specific for the types of collagen present in the matrices. We found increased expression of MMP-1 and partly MMP-2, but not of MMP-9 or MMP-13.
This finding is in keeping with published data, showing that fibroblasts grown on 3D collagen express MMP-1 and MMP-2, but not MMP-9 (18). Increase in MMP-2 levels has been suggested to stimulate the migratory activity of fibroblasts during the proliferative phase of wound healing as well as to increase angiogenesis (19).
Expression of TIMP genes, in particular TIMP-1 which targets MMP-1, was only slightly increased at 30 days in fibroblasts grown on all types of biological scaffolds, suggesting that the expressed metalloproteinases were enabled to exert their function. This suggested that in the time period under investigation, matrix degradation exceeded collagen biosynthesis and degradation was not (yet) inhibited by TIMP production (Figure 7). Protease activity could have the double role of: i) degrading collagen to be substituted by newly synthesized matrix; ii) allowing cells to penetrate into the scaffolds.
It has been shown that MMPs and TIMPs expression is regulated by several cytokines during wound healing (20,21) and that IL-6 in particular is involved in fibroblast proliferation and fibroproliferative disorders (22). These molecules are primarily produced by monocytes/macrophages at the wound site, however there is also evidence of an autocrine production by fibroblasts, especially during fibrosis (23,24). We tested autocrine production of IL-6 by fibroblasts grown on the different types of scaffold and found an increase in IL-6 transcript only when fibroblasts were grown on the Biodesign scaffold for 30 days. At this time, IL-6 protein was increased in fibroblasts grown in presence of all biological scaffolds, in spite of no apparent increase in transcript for Strattice and Permacol samples.
Finally, expression of CTGF and alpha-SMA was analyzed in order to evaluate the amount of fibrosis, that is strictly related to the amount of the cellular foreign body reaction (FBR) induced at the biomaterial/host-tissue interface 7. α-SMA, product of the ACTA2 gene, is considered a marker of fibroblast differentiation into myofibroblast during tissue remodelling and fibrosis (25). Its expression has been associated to up-regulation of collagen I and fibronectin biosynthesis and down-regulation of matrix remodelling enzymes (18).
Connective Tissue Growth Factor (CTGF) is a cytokine involved in adhesion, migration and proliferation of fibroblasts, but its overexpression is also involved in myofibroblast differentiation and fibrotic disease (26,27).
In particular, myofibroblast differentiation of fibroblasts during fibrosis is identified by the co-expression of collagen I and alpha-SMA (28,29), but in our study fibroblasts grown on biological matrices did not show expression of neither of these markers in the time frame under examination. It would have been interesting to compare expression of the same genes in cells grown on the synthetic mesh, but unfortunately we did not retrieve sufficient cells for molecular analyses. The synthetic mesh we used is a heavyweight polypropylene scaffold (Prolene). It is increasingly recognized that this type of scaffold can increase recurrence rates and comorbidities, including fibrosis, compared to more modern lightweight meshes (30). It would be interesting to see if use of the latter type of mesh improved cell attachment and proliferation. Our preliminary results suggest that this may indeed be the case, warranting further analyses of the potential differentiation of fibroblasts into myofibroblasts.
Our gene expression analyses suggest that the first transcriptional program activated by fibroblasts grown on biological matrices used for hernia repair involves expression of matrix degrading enzymes, independently of the tissue of origin or of the presence of crosslinking. It has been suggested that crosslinking may interfere with mesh incorporation in vivo (31-33), but no evidence

of a different behavior of cross-linked versus non cross-linked was found in our in vitro data. Taken together, our data suggest that, with time, all biological meshes are likely absorbed and substituted by endogenously produced extracellular matrix. Integration of data from in vitro and in vivo work (both in animal models and from human explants) is required to understand the interaction between host cells and different types of mesh, to be able to choose the right material for each case.

Conclusion

Cell growing on Strattice mesh was quantitatively inferior for number at 10 and 20 days respect to the other biological meshes, but anyway it was increasing by time and, at 30 days, it was superior to other biological meshes. Fibroblast seemed to be healthy and proliferating and their distribution was uniform. Cell appeared to be adherent on the matrix surface as confirmed by the number of cells retrieved for cell counting (cells were not lost during washes). Morphologically production of new extracellular matrix seemed occur, although this was not confirmed by gene expression analyses.

On Permacol mesh, cells were present early in larger number than on Strattice mesh but they were distributed unequally and seemed to interact weakly to the mesh surface, so that they were lost during wash step for sample preparation for cell counting.

The Surgisis-Biodesign mesh presented the largest number of cells, in particularly at early times, but cells seemed to interact strongly to each other, but more loosely to the matrix surface and they detached from it during matrix preparation for SEM analyses.

Cells did not appear to attached and grow easily on Prolene mesh and an adequate number of cells was not obtain with any of the methods used in cell harvesting.

Longer in vitro studies would be needed to better observe the remodeling phenomena and better understand the differences between the different matrices.

Preliminary "in vivo" studies

Successful regeneration requires precise coordination of multiple processes, which include scavenging cellular debris, proliferation and activation of progenitor cells, immune modulation, angiogenesis, and innervation of the newly forming tissue. The innate immune response serves not only to eliminate infections following injury but also to maintain homeostasis and functional integrity, and may be active in restoring structure to damaged tissues (35-37). Recent data suggest that efficient clearance of cellular debris by macrophages prevents the persistence of potentially toxic or immunogenic material in the tissue environment and promotes tissue regeneration. The integrity of tissue is restored by epithelial cells or fibroblasts proliferation and a new blood formation. These processes are stimulated by cytokines produced by inflammatory cells. Thus, the generation of a rapid inflammatory response plays different roles in both host defense and tissue repair.

Given the fundamental importance of innate immunity in both defense against pathogens and regeneration, innovative studies are necessary to give novel insight in how immune-mediated debris clearance and regenerative process are linked and to answer the important question relative to how the immune system can be both friend and foe to the damaged tissue. In wound healing research, it is well known that the employ of animal models is crucial as they provide a means of studying the complex interactions that occur in living tissues. Currently, many researchers use rodent or pig in wound healing studies but the number of animal species to use for experimentation has been recently reduced likely due to ethical considerations, stricter controls, improvements in animal welfare, etc.

Here we propose the use of the invertebrate medicinal leech, *Hirudo verbana*, as a powerful model system to gain novel insight into the basic mechanisms of the innate immune response and regenerative process. The rational of using this invertebrate is that has already been widely used for the study of tissue regeneration as it presents very simple anatomical features that facilitate the interpretation of data. Furthermore, the responses to wounds and grafts are very similar to those of vertebrates (38-41).

The focus of this part of the project, which has been performed under the direction of Prof. Annalisa Grimaldi of the Biotechnologies and Life Science Department in the University of Insubria, is to describe morphologically the *in vivo* interactions between the leech tissues and the same matrices used in "*in vitro"* experiments.

Animal model:

The leech, an invertebrate suitable for experimental manipulation, very economic, easily treated and without significant emotional factors related to use and regulatory restrictions, constitutes an interesting model for several reasons:

a) the different steps of tissue repair in leech are clear and easily detectable because they involve the whole thickness of body wall, which is virtually avascular in normal conditions and especially made of muscle fibers surrounded by loose connective tissue. This feature allows unambiguous assessment of the different wound healing steps (viz. inflammatory, angiogenic, fibroblastic and remodeling activities). Leeches are a good tool because any responses evoked by different stimuli are clear and easily detectable due to their small size and anatomical simplicity.

b) In leech surgically-stimulated, the accomplishments of the different processes linked to wound healing are fast: in 24 hours the entire target tissues of the animal are involved. This feature could be ideal also for rapid screening of large numbers of molecules active in repair tissues (i.e. anti/pro-inflammatory molecules, anti/pro-angiogenic, and anti/pro-fibroblastic factors)

c) the response of Hirudo to a variety of treatments (growth factor injections, surgical wounding) suggests that both surgical and biochemical stimuli may evoke vascular precursor cells signalling pathways analogous or similar to those observed in mammalians due to inflammatory response.

Taken together all these previous studies clearly demonstrate the surprising similarity among leech and vertebrates with regard to the cells involved in innate immune response such as macrophages, and those involved in wound healing and regeneration processes, such as fibroblasts and myofibroblasts.
The novel information could be useful to discover the roles of immune system and regenerative processes in integrating or rejecting the new transplanted matrices.

Material and method

Animal treatment:
Leeches (*Hirudo verbana*, Annelida, Hirudinea, from Ricarimpex, Eysines, France) were kept in lightly salted water (NaCl 1,5 g/l) at 19-20°C in aerated tanks. Animals were randomly divided into 4 separate experimental groups. Each group was composed of 3 individuals for each time point 10, 20, 30 days. Surgical grafting were performed on leeches anaesthetized by immersion in a 10% ethanol solution. On the surface of the 20th metamere, an incision of about 4-5 mm was made with a sterile razor blade. Then small pieces (about 4 mm) of the different matrices (Strattice, Permacol, Biodesign, Prolene) were inserted into the subcutaneous region. Grafts were sutured with was sutured with surgical Ethilon 5/0 (Polyamide 6 by Ethicon) to avoid transplant loss due to contraction of the highly muscular body wall.
Grafted leeches were kept in moist chambers for a post-surgical recovery period (of about 15-20 min), and subsequently placed in water tanks. The rate of successful transplantation experiments for all graft types was 90%. All leeches survived surgery and were able to move and feed following recovery from anaesthesia.
After 10, 20, 30 days animals were anesthetized as described above and implanted matrices were extracted from the body wall tissues and processed for electron scanning microscopy (SEM).
As a control condition, pieces of each type of sterile matrices (about 4 mm), were processed for SEM analysis.

Scanning electron microscopy (SEM) protocol:
To obtain 3D imaging by scanning electron microscopy (SEM), samples were fixed with Karnovsky fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate Buffer (pH7.2)) for 1 h at room temperature. Specimens, washed in 0.1 M cacodylate buffer (pH 7.2), were postfixed in a solution of 1% osmium tetroxide and potassium ferrocyanide for 2 h. Each specimen was washed in PBS (pH 7.2) and then immersed in 0.1% osmium tetroxide in PBS for 1 h. Samples were dehydrated in an increasing series of ethanol (70%, 90%, 100%), and subjected to critical point drying with hexamethyldisilazane. Dried samples were mounted on stubs, gold coated with a Sputter K250 coater (Emitech), and then observed with a SEM-FEG XL-30 microscope (Philips, Eindhoven, The Netherlands).

Results and Conclusions
Already 10 days after implantation (Fig. 10 B, F, J, N, N1) the four different types of implanted matrices were colonized by cells characterized by pseudopods and showing the typical appearance of macrophages. The deposition of new collagen was also visible.
After 20 (Fig. 10 C, G, K, O) and 30 days (Fig. 10 D, H, L, P) from the implant, a progressive increase in the deposition of collagen fibers was observed. In particular, after 30 days all the different types of matrices appear combined in a dense network made up of cells and of a newly synthesized collagenous material.
As expected, cells and newly synthesized collagen were not visible in the sterile matrices used as a control (Fig.10 A, E, I, M).

Our preliminary data confirmed that leeches respond to surgical lesions and grafts with the same sequence of events as that described for wound healing in vertebrates. Moreover, although the cells involved in these processes must be better characterized, the presence of macrophages suggested that they not only were involved in the recognition of non-self, but also in the recruitment of fibroblasts by producing specific cytokines and growth factors (42-43). In turn, fibroblasts were involved in the production of collagen fibrils, important for the development of tensions in wounds healing and functioning as an extracellular scaffold for accurate regeneration of the structures disrupted by surgical or traumatic events (43).

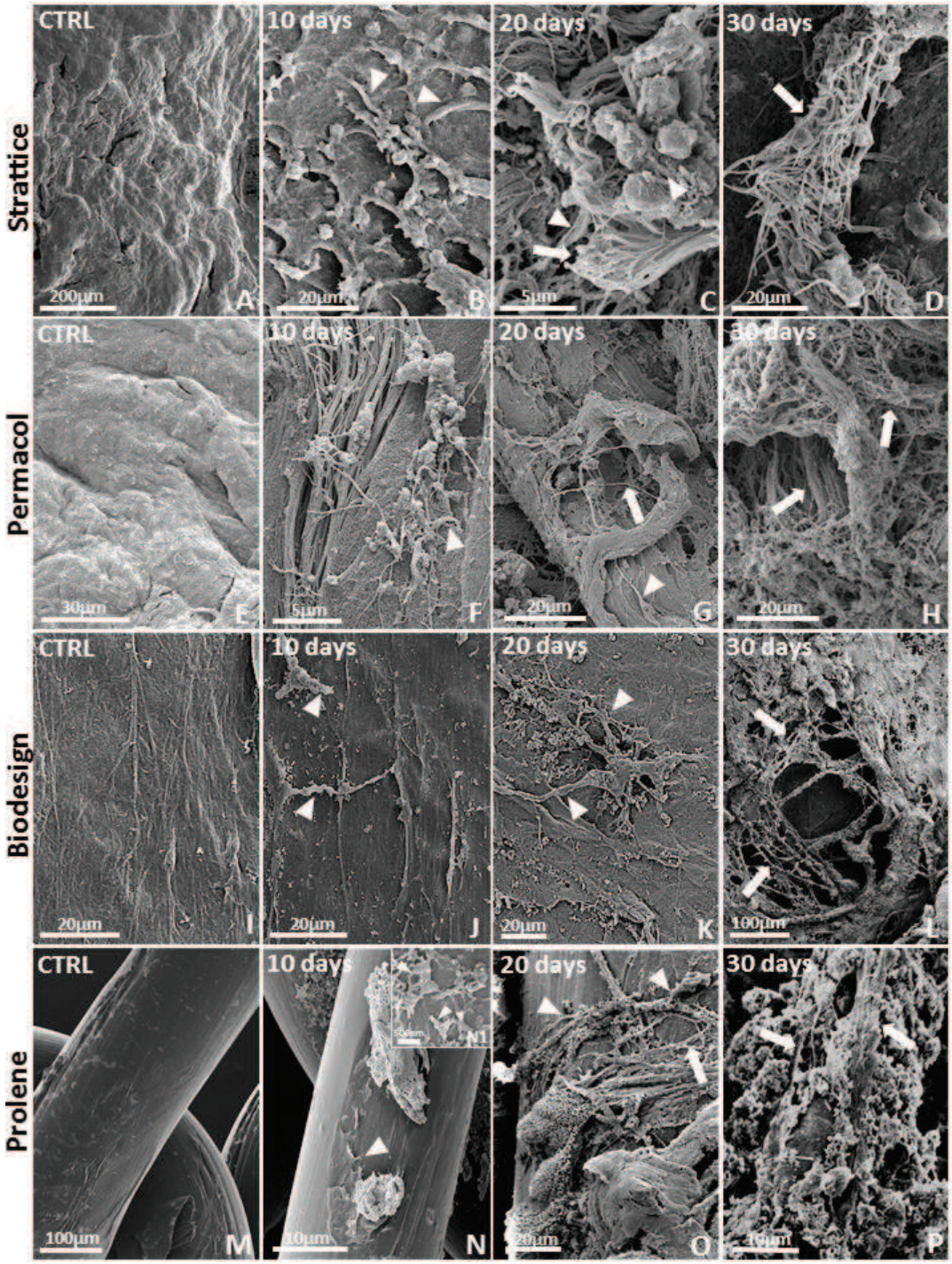


Fig. 10 *SEM images of different control and implanted matrices. After 10 and 20 days from grafts, macrophages-like cells characterized by pseudopodia (arrowheads) are recognizable on the surface of implanted matrices. After 20 and 30 days from the implant the deposition of collagen fibers was observed (arrows). After 30 days, the implants are completely covered by a network of cells and collagenous material (arrows).*

Clinical experience

Material and method

We collected all the data about our experience in biological scaffold in abdominal wall repair, in the last eleven years.
Since November 2007 to July 2018, we implanted 65 biological prosthesis in 63 patients: 24 Surgisis-Biodesign Cook (not crosslinked porcine intestinal submucosa), 21 Strattice Lifecell (non-crosslinked acellularized porcine derma), 12 Tutopatch Tutogen (non-crosslinked bovine pericardium), 2 PeriGuard Synovis (non-crosslinked bovine pericardium), 2 SurgiMend Tei (non-crosslinked fetal bovine derma), 4 Fortiva (non-crosslinked acellularized porcine derma).
Patients age ranged between 7 and 81 years.
Indication for use of biological mesh are listed in tab. 3.
Limited to groin hernia repair, we collect 15 primary inguinal hernia repairs in adults, 2 primary inguinal hernia repair in children, one incarcerate primary femoral hernia in adult, one primary femoral hernia in child. Two adult in inguinal hernia repair groups were under immunodepressing therapy, one after liver transplant and the second under corticosteroid therapy for rheumatoid arthritis.
In one patient with an indirect inguinal hernia (L2, according EHS classification (44)), a BioA plug (synthetic absorbable, polyglycolic acid-trimethylene carbonate) was placed in the inguinal ring to fill the defect and a Strattice mesh was then placed as reinforcement of the posterior inguinal wall.
We treated one prosthesis infection after plug and mesh inguinal hernia repair with the complete removal of both prosthesis and the placement of a biological (Strattice) mesh in the preperitoneal space.
We placed a biological scaffold during surgery for vas resection, even if a real hernia defect was not present, for the reinforcement of the inguinal posterior wall, in consideration of the opening of the external oblique aponeurosis to reach the cord.
We reported 11 Pubic Inguinal Pain Syndrome (45) treatment, the so called ”sports” hernia. In this kind of patient we are usually to propose a tailored open approach, under local anaesthesia, including nerve release, partial calibrated tenotomy of adductor longus and rectus abdominal muscles and reinforcement of the inguinal channel posterior wall with biological or lightweight mesh, as described in previous papers (45) (46).
We collected 7 post-operative inguinal chronic pain treatments with biological scaffolds: as reported in previous paper (47), in patient suffering by a chronic post-operative inguinal pain, we are used to approach the preperitoneal space in order to do the triple neurectomy (ilio-inguinal, ilio-hypogastric and genitofemoral nerve) and remove the plug previously placed, if present; then, through the same incision, we approach the anterior region to remove the mesh previously placed. We completed surgery with the placement of an ultralight or biological mesh in the preperitoneal space.
Regarding abdominal wall hernia repair, we reported one umbilical hernia repair, one incisional hernia repair after Pfannenstiel incision associated to a biopsy of endometrial nodule, three ventral hernia repairs associated to cosmetic surgery, 19 complex incisional hernia repairs and one chronic post-operative ventral pain treatment.

In the umbilical hernia repair the hernia defect was inferior to 1 cm and the mesh was placed in inlay position.
In the incisional hernia repair after Pfannenstiel incision, accomplished the biopsy of an endometrial nodule, the mesh was placed in the preperitoneal space and fixed with fibrin glue.
In the three patients with concomitant plastic surgery, the surgical steps were the following:

- first step, by plastic surgeon: sovrapubic transversal incision between the two anterior superior iliac spines, round incision around the umbilicus and elevation of the abdominal subcutaneous until the xyphoid region;
- the second step, by us: opening of the rectus sheet and of the abdominal cavity, isolation and reduction of hernia, preparation of a retromuscolar-preperitoneal space until the lateral edge of rectus muscle, accomplished of posterior component separation if necessary, closure of posterior rectus sheet, placement of the biological mesh fixed with fibrin glue sprayed on all the mesh surface (the umbilicus passed through a small incision in the mesh) and closure of the anterior rectus aponeurosis, keeping the umbilicus outside of the suture;
- The third step, by plastic surgeon: abdominoplasty with the excision of skin excess and replace of the umbilicus;

In 18 patients with incisional hernia and loss of substance, the biological mesh was bridged to the peritoneal sheet and placed in order to cover or complete the thin and weak peritoneal layer. A retromuscolar-preperitoneal space until the lateral edge of rectus muscle was prepared and a posterior component separation and, if necessary, a TAR (Transversus Abdominis Release) was accomplished. The synthetic mesh was then placed in the prepared space, on the top of the biological mesh, and fixed with fibrin glue sprayed on all the mesh surface.
In two patients, presenting a small recurrent incisional hernia and infection of the mesh previously placed (in one patient the infection was resolved at time of our surgery, in the second infection was still ongoing), the prosthesis was removed, component separation was accomplished, peritoneum and posterior rectus muscle sheets were closed and biological mash was placed in a retromuscolar-preperitoneal space.
In one patient, complying chronic pain after prosthetic ventral hernia repair, the mesh previously placed was removed and a biological mesh was placed in a retromuscolar-preperitoneal space, fixed with fibrin glue.
See table 3 for major details.

Table 3 Indication for biological mesh use in our experience in 56 patients

Indication for use of biological mesh	Number of patient treated with biological graft	Number of patient treated with biological graft plus synthetic not-absorbable mesh	Number of patient treated with biological graft plus synthetic absorbable mesh
Request by patient in primary inguinal hernia repair	11 divided in: 1 Strattice 6 Surgisis-Biodesign 1 Tutopatch 2 Peri-Guard 1 Fortiva	-	1 Strattice plus BioA plug Gore
Small indirect inguinal hernia	1 1 Surgisis	-	-
Inguinal hernia repair in patient under immunodepressed therapy	2 divided in: 1 Surgisis-Biodesign 1 Strattice	-	-
Inguinal hernia repair in children	2 2 Surgisis-Biodesign	-	-
Prosthesis and plug infection after inguinal hernia repair	1 1 Strattice	-	-
Incarcerated femoral hernia repair	1 1 Strattice	-	-
Femoral hernia repair in children	1 1 Tutopatch	-	-
Pubic Inguinal Pain Syndrome	11 divided in: 2 Strattice 4 Surgisis-Biodesign 4 Tutopatch 1 SurgiMend	-	-
Vas resection requested by the patient (in absence of inguinal hernia)	1 1 Surgisis	-	-
Postoperative inguinal chronic pain	7 divided in: 3 Tutopatch 3 Surgisis-Biodesign 1 Strattice	-	-

Small umbilical hernia	1 1 Surgisis	-	-
Small abdominal hernia defect repair with associated plastic surgery	3 divided in: 1 Strattice 2 Surgisis	-	-
Small incisional hernia and endometrial nodule biopsy	1 1 Tutopatch	-	-
Incisional hernia in infected field	1 1 Surgisis-Biodesign	-	-
Incisional hernia in previous infection	1 1 SurgiMend	-	-
Loss of substance in incisional hernia repair	-	13 divided in: 7 Strattice plus polypropylene 1 Strattice plus PVDF 2 Tutopatch plus polypropylene 3 Fortiva plus polypropylene	-
Loss of substance and previous infection/contaminated field in incisional hernia repair	-	3 3 Strattice plus polypropylene	-
Loss of substance and contaminated field in incisional hernia repair	-	2 divided in: 1 Strattice plus polypropylene 1 Surgisis-Biodesign pus polypropylene	-
Postoperative ventral chronic pain	1 1 Surgisis-Biodesign	-	-
Total	46	18	1

Results

No complications during intraoperative time were reported.
One patient, underwent to abdominal repair with Strattice mesh and synthetic (Hermesh, polypropylene) for an incisional medial and iliac hernia (site of previous ileostomy), required surgery in 11th day for a dehiscence of bowel lesion and both meshes were removed.
One deep wound infection requiring VAC therapy for two months was reported in a patient underwent to removal of previous infected mesh, component separation and biological repair (Surgisis).
Follow up ranges from 4 to 132 months.
Two inguinal recurrences were recorded in two patients underwent to Lichtenstein modified repair with Surgisis-Biodesing, respectively two and four years after surgery.
Patient suffering with PIPS treated with biological mesh reported 100% pain relief.
Regarding patients treated for chronic post-operative pain, pain relief was obtain in 4 patient and one patient reported hernia recurrence 3 years after surgery (Tutopatch).
One patient with incisional hernia with loss of substance, treated with biological plus synthetic not-absorbable mesh, died 3 years after surgery for abdominal aortic aneurysm rupture.
Three early seroma were found after abdominal wall reconstruction, one with Strattice and two with Fortiva, located in the space between the prosthesis (biologic and syntetic not-absorbable) and in the subcutaneous space close to the wound; one required surgical drainage. Among these three patients, one was affected by severe obesity, the other two were overweight.
Two superficial wound infections, both treated with incision and antibiotics, have been reported: one after complex incisional hernia repair with Fortiva, the other after inguinal hernia repair with Strattice in a previously contaminated field.

Discussion and conclusions

Despite our experience includes 65 procedures in 63 patients, the variety in indications and kind of surgeries makes difficult to compare our results with those from other series.
Limited to inguinal hernia repair, in literature two meta-analysis (48) (49) of respectively three (50) (51) (52) and four (52) (53) (54) (55) RCTs, comparing the use of biological (Surgisis-Biodesign and Strattice) and synthetic meshes, are present. No difference was found in the recurrence and pain rate. Only the discomfort rate was lower in the Surgisis-Biodesign group, but the seroma rate was higher in this group (48) (49).
In two retrospective case series (49) with respectively 11 and 38 patients, inguinal hernias were repaired in an endoscopic technique (respectively TAPP and TEP) with Surgisis-Biodesign. During the follow-up period (1-30 months), a recurrence rate of 9.1 and 2% was observed, respectively. In another study the biological meshes (Surgisis-Biodesign) were used successfully even in a potentially contaminated setting, i.e., with incarcerated/strangulated bowel within the hernia or coincident with a laparoscopic cholecystectomy/colectomy as well as in a grossly contaminated field (i.e., gross pus or fecal spillage) (19).
However, the median follow-up is rather limited; just one RCT (51) reported results 3 years after surgery. Bochicchio et al. (52) reported a higher recurrence rate (6,7%, 3/45 patients), not statistically significant, in biologic mesh (Surgisis-Biodesign) group at 1-year follow-up. They attributed the high recurrence rate to the inexperience of the surgeons and suggested that a biologic repair might require some added technical skill and experience.

	Puccio *et al.*	Ansaloni *et al.*	Bochicchio *et al.*	Bellows *et al.*
Year	2005	2003 and 2009	2014	2014
Country	Italy	Italy	United States	United States
Mean age				
BM	54	61.3	64	56
SM	53.5	56.2	59	57
Patients				
BM	15	35	45	84
SM	30	35	50	88
Gender	Male (≥18 years)	Male (≥18 years)	Male (≥18 years)	Male (≥18 years)
Median follow-up (months)	3	36	12	3
Type of hernia	Primary and unilateral	Primary and unilateral	Primary and unilateral	Primary and unilateral
Surgery technique	Lichtenstein	Lichtenstein	Lichtenstein	Lichtenstein
Type of biologic mesh (Maker)	Surgisis (Cook)	Surgisis (Cook)	Biodesign (Cook)	Strattice (Life cell)
Type of synthetic mesh (Maker)	PP and Vppro (NG)	PP (Angiologica)	PP (NG)	Ultrapro (Ethicon)
Recurrence				
BM	0	0	3	0
SM	0	1	0	0
Chronic groin pain				
BM	0	2	2	13
SM	1	7	3	20
Seroma				
BM	0	6	5	6
SM	1	2	0	4
Hematoma				
BM	0	2	6	7
SM	3	2	1	5
Operating time (min)				
BM	NG	66.6 ± 13.7	134	74.0 ± 30.5
SM	NG	66.0 ± 20.4	115	64.2 ± 22.0

BM, biologic mesh; NG, not given; PP, polypropylene; SM, synthetic mesh.

We reported two recurrences (25%, 2/8 patients) in adult patients underwent to inguinal hernia repair with Surgisis-Biodesign mesh, respectively at two and four years after surgery.

As previously said, it is not possible to compare our results considering the limited number in our series, but anyway our experience is proof that more extensive study with longer follow-up period is necessary.

Catena et. al (56) reported their experience in inguinal hernia repair with Surgisis-Biodesign mesh in ten immunodepressed patients with good results: no wound infection, no recurrence. We confirm that the use of biological mesh is a good indication in immunodepressed patients.

We reported one case of incarcerate femoral hernia: the crural sac passed through femoral vein and artery and, after sac reduction by transinguinal preperitoneal approach, we preferred to place a biological (Strattice) prosthesis in direct contact with femoral vessels, rather synthetic mesh in order to prevent potential complication, like chronic pain, adhesion and erosion. No intra or post-operative complication were reported. At 18 months after surgery, patient does not complain chronic pain neither recurrence.

For the same reason, we adopted a biological mesh repair in a 7 years old-child.

We collected two cases of inguinal hernia repair in children (14 and 17 years old) with Surgisis-Biodesign mesh: in these patients we chose a biological repair because, like in all teenager, their body appearance were not typically of children, but even of adults, so a pure tissue repair seemed not suitable like normally in children, but even a prosthetic repair seemed not correct due to the incomplete physical development. No complications were observed.

No data about biological mesh use are available in literature.

Regarding patients affected by Pubic Inguinal Pain Syndrome (45), the so called ”sports” hernia, we proposed a tailored open approach (45) (46), including nerve release, partial calibrated tenotomy of adductor longus and rectus abdominal muscles and reinforcement of the inguinal channel posterior wall with biological mesh. Edelman proposed a laparoscopic TEP approach and the placement of a biological Surgisis-Biodesign mesh fixed with absorbable tacks and, through a separate skin incision along the inguinal crease, micro-cuts of the tendon of the adductor muscle and the placement of a biological mesh tacked to the inferior pubis and sutured to the adductor muscle.

The use of a biological mesh is indicated by the absence of a real hernia defect. Normally, just a bulge of the posterior wall and a small indirect sac (M1,L1, according EHS classification (44)) are present, contributing to compress the nerves against the hypertrophic rectus muscle. The open approach under local anaesthesia permits to ask the patient to do easily exercises during surgery and so to calibrate the partial tenotomy. By a single incision in inguinal region, we can reach inguinal channel, rectus muscle and adductor longus muscle and so act on all the reason of pain.
We adopt the use of biological mesh also in the treatment of the post-operative inguinal pain (47): in these patients, the removal of the prosthesis previously placed can caused a weakness of the transversalis fascia and, sometimes, of the external oblique aponeurosis in absence of a real hernia, so the placement of a mesh has a precautionary aim. We prefer a biological mesh or an ultralight one to reduce the discomfort that a heavyweight mesh can caused.
In ventral hernia repair, we always adopt biological mesh to fill the gap when a real loss of substance is seen and a further synthetic mesh to complete the abdominal wall reconstruction. Campanelli et al. (57) (58), when biological mesh use was at beginning and very restricted, reported good results in the "double mesh technique", with the use of vicryl (absorbable polyglactin mesh) mesh bridged to peritoneum and posterior sheet aponeurosis to fill the loss of substance and isolate the viscera from the synthetic mesh placed in the retromuscolar-preperitoneal space.
A recent paper by Liu (59), utilization of the absorbable polyglactin mesh, as a separating layer between a synthetic mesh and viscera in a porcine model, was found to be associated with similar adhesion formation as unprotected synthetic meshes grossly. Histologically, however, visceral adhesions formed not against the synthetic mesh, but against a fibrous capsule that replaced vicryl mesh and probably this capsule could prevent intestinal erosions into retromuscular synthetic meshes.
Since biological scaffolds became more popular, we prefer them to vicryl mesh because adhesions should not occur with biological scaffold.
We chose to implant a biological mesh alone in all those patients reporting small ventral hernia, in absence of abdominal wall relaxation or diastasis recti.
Moreover, we confirmed that the use of synthetic mesh is feasible also in contaminated or potentially contaminated field, as reported in a previous study (58).
Bioprosthetic mesh materials clearly have certain advantages over other implantable mesh materials in select indications. Appropriate patient selection and surgical technique are critical to the successful use of bioprosthetic materials for abdominal wall repair.
Studies with longer follow-ups are essential to properly determine the durability of biologic grafts given their biodegradable nature.

References

1. **IM, Rutkow.** Surgical procedure in the United States. Then (1983) and now (1994). *Arch Surg 1997, 132(9):983–990.*

2. **Engelsman AF, van der Mei H, Ploeg RJ, Busscher HJ.** The phenomenon of infection in abdominal wall reconstruction. *Biomaterials 2007,28(14):2314–2327.*

3. **Ansaloni L, Catena F, Coccolini F, Negro P, Campanelli G, Miserez M.** New"biological" meshes: the need for a register, The EHS Registry forBiological Prostheses: call for participating European surgeons. *Hernia 2009, 13(1):103–108.*

4. **Uscher FC.** Hernia repair with marlex mesh. An analysis of 514 cases. *Arch Surg 1962, 84:325–328.*

5. **Halm JA, de Wall LL, Steyerberg EW, Jeekel J, Lange LW.** Intraperitoneal polyproylene mesh repair complicates subsequent abdominal surgery. *World J Surg 2007: 31:423–431.*

6. **Gray SH, Vick CC, Graham LA, Finan KR, Neumayer LA, Hawn MT.** Risk of complications from enterotomy or unplanned bowel resection during elective hernia repair. *Arch Surg 2008: 143(6):582–586.*

7. **Kissane N, Itani KMF.** A decade of ventral incisional hernia repairs with biologic acellular dermal matrix: what have we learned? *Plast Reconstr Surg 2012: 130:194S–202S.*

8. **Cevasco M, Itani KM.** Ventral hernia repair with synthetic composite and biologic mesh: characteristics, indications and infection profile. *Surg Infect 2012: 13:209–215.*

9. **Rosen MJ, Krpata DM, Ermlich B, Blatnik J.** A 5-year clinical experience with single-staged repairs of infected and contaminated abdominal wall defects utilising biologic mesh. *Ann Surg 2013: 257:991–996.*

10. **De Castro Bras LE, Shurey S, Sibbons PD.** Evaluation of crosslinked and non-crosslinked biologic prostheses for abdominal hernia repair. *Hernia 2012;16:77–89.*

11. **Pierce LM, Rao A, Baumann SS Glassberg JE, Kuehl TJ, Muir TW.** Long-term histologic response to synthetic and biologic graft materials implanted in the vagina and abdomen of a rabbit model. *Am J Obstet Gynecol. 2009;200:e541–e548.*

12. **Slater NJ, van der Kolk Hendriks T, van Goor H, Bleichrodt RP.** Biologic grafts for ventral hernia repair: a systematic review. *Am J Surg 2013: 205:220–230.*

13. **Muysoms F, Campanelli G, Champault GG, DeBeaux AC, Dietz UA, Jeekel J, Klinge U, Kockerling F, Mandala V, Montgomery A, Morales Conde S, Puppe F, Simmermacher RKJ, Smietanski M, Miserez M.** EuraHS: the development of an international online platform for registration and outcome measurement of ventral abdominal wall hernia repair. *Hernia 2012: 16:239–250.*

14. **Hamer-Hodges DW, Scott NB.** Surgeon's workshop:replacement of an abdominal wall defect using expanded PTFE sheet (GORE-TEX). *J Royal Coll Surg Edin 1985: 30:65–67.*

15. **McPherson TB, Badylak SF.** Characterization of fibronectin derived from porcine small intestinal submucosa. *Tissue Eng. 1998;4(1):75–83.*

16. **Hodde JP, Badylak SF, Brightman AO, Voytik-Harbin SL.** Glycosaminoglycan concontent of small intestinal submucosa: a bioscaffold for tissue replacement. *Tissue Eng 1996;2(3):209–17.*

17. **Hodde JP, Ernst DMJ, Hiles MC.** An investigation of the long- term bioactivity of endogenous growth factor in OASIS Wound Matrix. *J Wound Care. 2005;14(1):23–5.*

18. **McDevitt CA, Wildey GM, Cutrone RM.** Transforming growth factor-B1 in a sterilized tissue derived from the pig small intestine submucosa. *J Biomed Mater Res 2003;67(2):637–40.*

19. **Gaertner WB, Bonsack ME, Delaney JP.** Experimental evaluation of four biologic prostheses for ventral hernia repair. *J Gastrointest Surg. 2007;11:1275–1285.*

20. **Cornwell KG, Landsman A, James KS.** Extracellular matrix biomaterials for soft tissue repair. *Clin Podiatr Med Surg. 2009; 26:507–523.*

21. **Sandor M, Xu H, Connor J, et al.** Host response to implanted porcine-derived biologic materials in a primate model of abdominal wall repair. *Tissue Eng Part A. 2008;14:2021–2031.*

22. **Khor E.** Methods for the treatment of collagenous tissues for bioprostheses. *Biomaterials 1997;18:95–105.*

23. **Schmidt CE, Baier JM.** Acellular vascular tissues: Natural biomaterials for tissue repair and tissue engineering. *Biomaterials 2000;21:2215–2231.*

24. **Deeken CR, Melman L, Jenkins ED, Greco SC, Frisella MM, Matthews BD.** Histologic and biomechanical evaluation of crosslinked and non-crosslinked biologic meshes in a porcine model of ventral incisional hernia repair. *J Am Coll Surg. 2011;212(5):880–8.*

25. **Gouk SS, Lim TM, Teoh SH, Sun WQ.** Alterations of human acellular tissue matrix by gamma irradiation: Histology, biomechanical property, stability, in vitro cell repopulation, and remodeling. *J Biomed Mater Res B Appl Biomater. 2008;84:.*

26. **Harth KC, Rosen MJ.** Major complications associated with xenograft biologic mesh implantation in abdominal wall reconstruction. *Surg Innov. 2009;16(4):324–9.*

27. **Petter-Puchner AH, Fortelny RH, Silic K, Brand J, Gruber-Blum S, Redl H.** Biologic hernia implants in experimental intraperitoneal onlay mesh plasty repair: The impact of proprietary collagen processing methods and fibrin sealant application on tissue integration. *Surg Endosc. 2011;25:3245–3252.*

28. **Butler CE, Burns NK, Campbell KT, Mathur AB, Jaffari MV, Rios CN.** Comparison of cross-linked and non-cross-linked porcine acellular dermal matrices for ventral hernia repair. *J Am Coll Surg. 2010;211:368–376.*

29. **Orenstein SB, Qiao Y, Klueh U, Kreutzer DL, Novitsky YW.** Activation of human mononuclear cells by porcine biologicmeshes in vitro. *Hernia 2010;14:401–407.*

30. **Novitsky YM, Rosen MJ.** The Biology of Biologics: Basic Science and clinical concepts. *Plast. Reconstr. Surg. 130 (Suppl. 2): 9S, 2012.*

31. **Xu H, Wan H, Sandor M, et al.** Host response to human acellular dermal matrix transplantation in a primate model of abdominal wall repair. *Tissue Eng Part A. 2008; 14:2009–2019.*

32. **Orenstein SB, Qiao Y, Kaur M, Klueh U, Kreutzer DL, Novitsky YW.** Human monocyte activation by biologic and biodegradable meshes in vitro. *Surg Endosc. 2010;24:805–811.*

33. **Burns NK, Jaffari MV, Rios CN, Mathur AB, Butler CE.** Non cross-linked porcine acellular dermal matrices for abdominal wall reconstruction. *Plast Reconstr Surg. 2010;125:167–176.*

34. **Melman L, Jenkins ED, Hamilton NA, et al.** Early biocompatibility of crosslinked and non-crosslinked biologic meshes in a porcine model of ventral hernia repair. *Hernia 2011;15:157–164.*

35. **Martin, J. T.** Development of an adjuvant to enhance the immune response to influenza vaccine in the elderly. Biologicals 25, 209–213 (1997).

36. **Saltzman CL** Total ankle arthroplasty: state of the art. Instr. Course Lect. 48, 263–8 (1999).

37. **Frantz S, Vincent KA, Feron O & Kelly RA** Innate immunity and angiogenesis. Circ. Res. 96, 15–26 (2005).

38. **Grimaldi A**: Origin and fate of hematopoietic stem precursor cells in the leech Hirudo medicinalis. Invertebr Surviv J 2016;13:257–268.

39. **Grimaldi A, Tettamanti G, de Eguileor M: Annelida**: Hirudinea (Leeches): Heterogeneity in Leech Immune Responses; in: Advances in Comparative Immunology. Cham, Springer International Publishing, 2018, pp 173–191.

40. **Tettamanti G, Grimaldi A, Ferrarese R, Palazzi M, Perletti G, Valvassori R, Cooper EL, Lanzavecchia G, De Eguileor M**. Leech responses to tissue transplantation. Tissue & Cell 35 (2003) 199–212.

41. **Tettamanti G, Grimaldi A, Congiu T, Perletti G, Raspanti M, Valvassori R and De Eguileor M.** Collagen reorganization in leech wound healing. Biol. Cell (2005) 97, 557–568.

42. **Schorn T, Drago F, de Eguileor M, Valvassori R, J Vizioli, Tettamanti G, Grimaldi A.** 2015. The Allograft Inflammatory Factor-1 (AIF-1) homologous in Hirudo medicinalis (medicinal leech) is involved in immune response during wound healing and graft rejection processes, Invert. Surv. J. 12: 129-141

43. **Baranzini, N. et al.** Human recombinant RNASET2-induced inflammatory response and connective tissue remodeling in the medicinal leech. Cell Tissue Res. 368, 337–351 (2017).

44. **Miserez M, Alexandre JH, Campanelli G, Corcione F, Cuccurullo D, Pascual MH, Hoeferlin A, Kingsnorth AN, Mandala V, Palot JP, Schumpelick V, Simmermacher RK, Stoppa R, Flament JB.** The European hernia society groin hernia classification: simple and easy to remember. *Hernia. 2007 Apr;11(2):113-6.*

45. **G., Campanelli.** Pubic inguinal pain syndrome: the so-called sports hernia. *Hernia. 2010 Feb;14(1):1-4.*

46. **Cavalli M, Bombini G, Campanelli G.** Pubic inguinal pain syndrome: the so-called sports hernia. *Surg Technol Int. 2014 Mar;24:189-94.*

47. **Campanelli G, Bertocchi V, Cavalli M, Bombini G, Biondi A, Tentorio T, Sfeclan C, Canziani M.** Surgical treatment of chronic pain after inguinal hernia repair. *Hernia. 2013 Jun;17(3):347-53.*

48. **Nie X, Xiao D, Wang W, Song Z, Yang Z, Chen Y, et al.** Comparison of porcine small intestinal submucosa versus polypropylene in open inguinal hernia repair: a systematic review and meta-analysis. *PLoSOne (2015) 10(8):e13507.*

49. **Fang Z, Ren F, Zhou J, Tian J.** Biologic mesh versus synthetic mesh in open inguinal hernia repair: system review and meta-analysis. *ANZ JSurg (2015).doi:10.1111/ans.13234.*

50. **Puccio F, Solazzo M, Marciano P.** Comparison of three different mesh materials in tension-free inguinal hernia repair: Prolene versus Vypro versus Surgisis. *Int. Surg. 2005; 90: S21–3.*

51. **Ansaloni L, Catena F, Coccolini F, Gazzotti F, D'Alessandro L, Pinna AD.** Inguinal hernia repair with porcine small intestine submucosa: 3-year follow-up results of a randomized controlled trial of Lichtenstein's repair with polypropylene mesh versus Surgisis Inguinal Hernia Matrix. *Am. J. Surg. 2009; 198: 303–12.*

52. **Bochicchio GV, Jain A, McGonigal K et al.** Biologic vs synthetic inguinal hernia repair: 1-year results of a randomized double-blinded trial. *J Am. Coll. Surg. 2014; 218: 751–7.*

53. **Bellows CF, Shadduck P, Helton WS, Martindale R, Stouch BC, Fitzgibbons R.** Early report of a randomized comparative clinical trial of Strattice reconstructive tissue matrix to lightweight synthetic mesh in the repair of inguinal hernias. *Hernia 2014; 18: 221–30.*

54. **Agresta F, Bedin N.** Transabdominal laparoscopic inguinal hernia repair: is there a place for biological mesh? *Hernia (2008) 12:609–12.*

*55. **Bellows CF, Shadduck PP, Helton WS, Fitzgibbons RJ.** The design of an industry-sponsored randomized controlled trial to compare synthetic mesh versus biologic mesh for inguinal hernia repair. Hernia, June, 2011, Vol.15(3), p.325-8).*

56. **Catena F, Ansaloni L, Leone A, De Cataldis A, Gagliardi S, Gazzotti F, Peruzzi S, Agrusti S, D'Alessandro L, Taffurelli M.** Lichtenstein repair of inguinal hernia with Surgisis inguinal hernia matrix soft-tissue graft in immunodepressed patients. *Hernia. 2005 Mar;9(1):29-31.*

57. **Campanelli G, Bastazza M, Ruca A, senni Buratti M, Casirani R, Nicolosi MF, Trivellini G, Pietri P.** Surgical treatment of incisional hernias with marked loss of substance. *Hernia (2000); 4:202-205.*

58. **Campanelli G, Nicolosi FM, Pettinari D, Avesani EC.** Prosthetic repair, intestinal resection, and potentially contaminated areas: safe and feasible? *Hernia. 2004 Aug;8(3):190-2.*

59. **Liu L, Petro C, Majumder A, Fayezizadeh M, Anderson J, Novitsky YW.** The use of Vicryl mesh in a porcine model to assess its safety as an adjunct to posterior fascial closure during retromuscular mesh placement. *Hernia. 2016 Apr;20(2):289-95.*

Abstract

Synthetic and biological matrices for abdominal wall repair have been developed and commercialized in recent years. Biological meshes have been proposed as an alternative when synthetic implants are not indicated, as in the case of contaminated surgical field and may present fewer complications such as chronic pain and visceral adhesions after hernia repair. However, their use is still debated, due to high cost to benefit ratio. Moreover, knowledge of the molecular pathways activated in the different types of cells by their use is still lacking.

This study aimed to investigate the molecular processes activated by fibroblasts during their interaction with different types of biological and synthetic matrices, comparing the fibroblast-matrix interactions morphologically, monitoring cell proliferation and the expression of genes involved in the deposition and reabsorption of collagen, as well as some cytokines and markers of differentiation into myofibroblasts.

We found that fibroblasts grew differently on the different biological meshes. Few fibroblasts grew on the synthetic mesh, impairing gene expression analysis. Fibroblasts on biological meshes induced specific metalloproteinases and reduced expression of collagen genes compared to control cells. Expression of markers for myofibroblast differentiation was also reduced. We found limited differences in gene expression programs among the different biological meshes.

Keywords

Abdominal wall repair; meshes; human dermal fibroblasts; gene expression; cell growth.