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Author(s):
Zaytseva, Polina; Visser, Valery L.; Ehterami, Arian; Hoerstrup, Simon P.; Motta, Sarah E.; Emmert, Maximilian Y.

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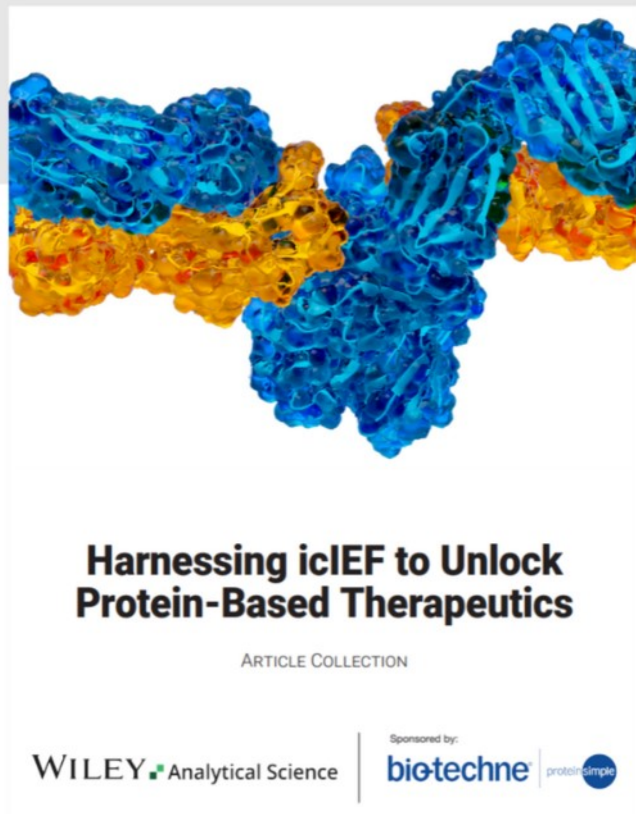

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Xenogeneic Serum-Free Human Cell-Derived Tissue Engineered Matrices for the Development of Clinical-Grade Biomimetic Cardiovascular Devices

Polina Zaytseva, Valery L. Visser, Arian Ehterami, Simon P. Hoerstrup, Sarah E. Motta, and Maximilian Y. Emmert**

The development of next-generation biomimetic cardiovascular implants using tissue engineering concepts can address the existing shortcomings of the clinically available prostheses, offering the possibility to generate life-long, native-analogous constructs with self-remodeling and regenerative capacities. Scaffolds for tissue-engineered cardiovascular prostheses can be obtained from allogenic cell sources, that can then produce human tissue-engineered matrices (hTEMs) in vitro. Traditionally, fetal bovine serum (FBS) is used as a universal cell growth supplement. However, concerns regarding its biosafety remain a challenge for clinical translation. The aim of this study is to develop a novel xenogeneic serum-free approach for the manufacturing of clinical grade hTEMs. To achieve this, decellularized hTEMs are generated under xenogeneic serum-free conditions and have subsequently demonstrated hTEMs perform similarly to the FBS-supplemented control group in terms of extracellular matrix (ECM) composition, hemocompatibility, thrombogenicity, and calcification potential. Finally, the xenogeneic serum-free protocol is successfully adapted to the development of hTEM-based tissue-engineered heart valves for the systemic circulation, showing proof-of-concept functionality in vitro. Overall, the data suggest the effectiveness of xenogeneic serum-free culture method as a valid alternative to FBS for the production of hTEM for cardiovascular applications.

1. Introduction

Cardiovascular diseases is one of the leading causes of death in the Western world[1] and the ageing population is contributing to the incidence rate increase of severe degenerative heart diseases. Current treatments involve the replacement of a damaged vessel or valve. Clinically available synthetic conduits, usually made of bioinert materials such as Dacron or Goretex, are long-lasting and provide sufficient functionality. On the other side, human- (homografts) and xenogeneic-based vascular and valvular replacement (made of bovine pericardium, arteries or porcine valves) are biological substitutes, capable of mimicking the physiological hemodynamical behavior of the native counterparts, while providing a relatively durable and mechanically stable prosthesis.[2]

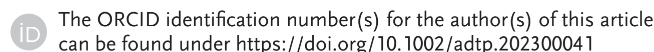
Nevertheless, currently available prostheses still suffer from severe drawbacks, such as limited durability and availability, thrombogenicity, immunogenicity, and eventually rejection within few months after surgery.[3] Particularly, synthetic and/or mechanical substitutes may require a lifelong anticoagulation therapy, thereby substantially affecting the life quality of the patients

P. Zaytseva, V. L. Visser, A. Ehterami, S. P. Hoerstrup, S. E. Motta, M. Y. Emmert
Institute for Regenerative Medicine
University of Zurich
Schlieren 8952, Switzerland
E-mail: sarahmotta@irem.uzh.ch; maximilian.emmert@irem.uzh.ch

S. P. Hoerstrup
Wyss Zurich
University of Zurich and ETH Zurich
Zurich 8092, Switzerland

M. Y. Emmert
Charité Universitätsmedizin
Berlin, Germany

M. Y. Emmert
Deutsches Herzzentrum der Charite (DHZC)
Department of Cardiothoracic and Vascular Surgery
13353 Berlin, Germany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and increasing the risk of major bleeding complications. Human homografts represent a promising alternative to synthetic and mechanical prostheses, given their anatomy, composition, hemodynamic, and reduced thrombogenic and immunogenic risks. However, their limited availability as well as incidence of early calcification onset still limit their broader clinical application.[4,5] Bioprostheses are composed of glutaraldehyde-fixed xenogeneic material being therefore prone to over time stiffening and altered mechanical functions, which ultimately results into calcification and degeneration. Despite the recent technological advances in the field of cardiovascular therapy with the implementation of minimally invasive transcatheter technologies, the clinical need for nonthrombogenic replacements, which do not calcify, and possess regenerative, self-repair, and growth potential capacities, is still unmet.

Next-generation in situ human cell-based tissue engineered matrices (hTEMs) may have the potential to overcome the shortcomings of existing prostheses and may represent a readily off-the-shelf-available life-long replacement, capable of harnessing the regenerative capacities of the host to remodel into a native-like tissue.[2] This approach relies on cells seeded on a biodegradable scaffold and cultured in a bioreactor to produce extracellular matrix (ECM), which is subsequently decellularized and ready for implantation.[6] The resulting construct must be capable to withstand physiological pressures upon implantation as well as support host cell adhesion and infiltration, adequate neotissue formation, and provide a less thrombogenic substitute with low calcification potential. TEM-based replacements have been used as substitutes for several indications such as pulmonary and aortic valves, occluders, and blood vessels in large animal models.[7–11] A proof-of-concept study demonstrated that TEM-based tissue-engineered heart valves could be used to replace the pulmonary valve in sheep in a 1-year follow-up study, demonstrating clinical grade in vivo performance together with native-like remodeling. Such approach has been recently translated to the aortic setting, where a human dermal fibroblast (hDF) derived hTEM successfully withstood aortic pressure conditions in a sheep model. Nevertheless, despite the promising approach of producing hTEMs with a systematic and reliable decellularization protocol, the aim should be to further optimize the manufacturing protocols toward the complete removal of xenogeneic antigens. This would firstly allow to enhance the overall translatability of such concepts, by eliminating potential negative remodeling phenomena due to such xenogeneic antigens, and thus ultimately strengthen the safety and predictability of hTEMs.

Culture media for cell culture and expansion usually comprise serum supplements.[12] Traditionally, fetal bovine serum (FBS) is used as universal growth supplement of tissue culture media, due to its high concentration of growth factors which promote cell attachment and proliferation. It also contains essential components, such as hormones and vitamins. FBS is effective for most types of mammalian cells[13] and is readily available. However, despite its advantages and its use for over 50 years, its composition has never been fully characterized.[13] There are several major drawbacks to the use of FBS in cell culture. First of all, FBS is an ill-defined cocktail with batch-to-batch variations, which could potentially decrease the product reproducibility during production. Additionally, FBS may be contaminated with zoonotic pathogens such as endotoxin, mycoplasma, bovine viral particles, or prion proteins,[13] or contain xenogeneic proteins (e.g., *N*-glycolylneuraminic acid), which can increase its immunogenicity.[14] Calls for the clinical substitution of animal-derived supplements for xenogeneic-free components have been made by the regulatory authorities.[15,16] In this context, the Committee for Proprietary Medical Products has been established under the European Agency for Evaluation of Medical Products (2003) and it recommends the replacement of FBS with nonanimal origin material or reduction of its use.[17] Thus, research on FBS substitutes in cell culture media, that allow the cells to grow and proliferate, while preserving their characteristics, has become increasingly relevant. Human platelet lysate (HPL) and human serum (HS) have been noted as a potential growth supplements and FBS substitutes, being allogeneic and rich in growth factors or human proteins, respectively, and showing reduced batch-to-batch variability.[18]

In the present study, we systematically developed a xenogeneic serum-free cell culture protocol based on HPL and HS as supplements to create a novel hTEM specifically designed for the systemic high-pressure circulation. To achieve this, we first performed a comparison between the FBS-supplemented (control) and the xenogeneic serum-free culture protocols by examining tissue morphology, protein content, as well as tissue characterization via histological, biochemical, and proteomic analyses. Thereafter, thrombogenicity and the risk for potential calcification onset were analyzed between control and xenogeneic serum-free hTEMs. Thereafter, the xenogeneic-serum free hTEM culture protocol was further adapted to create a tissue-engineered heart valve for the systemic circulation, which performance was assessed in vitro under ISO conditions. Taken together, our data suggest xenogeneic serum-free culture method as a valid alternative to FBS for the production of hTEM for cardiovascular applications.

2. Results

2.1. Influence of Medium Supplementation on hTEM Development

In order to develop a reliable and reproducible xenogeneic serum-free protocol, we first tested hTEMs culture methods under different conditions. Here, HPL-only and xenogeneic serum-free (HPL and HS) supplements were tested as FBS alternative to our cells and tissue cultures.

After 4 weeks, hTEMs cultured under control (FBS), HPL-only, and xenogeneic serum-free (HPL and HS) conditions (**Figure 1**) showed homogeneous neotissue formation with shiny and smooth ECM at the surface (**Figure 2**A–D). Histological analyses of the decellularized patches were used to assess the tissue morphology, as well as the presence and the distribution of ECM (Figure 2Bi-Biii, Ci-Ciii, Di-Diii). Collagen I and III stainings confirmed the presence of collagen layers. Particularly, HPL-only group was characterized by poorly organized tissue and sparce fibers (Figure 2Ci-Ciii), while in the xenogeneic serum-free group, dense collagen layers were observed through the transversal plane (Figure 2Di-Diii). Quantitative analyses for the content of glycosaminoglycans (GAGs) and hydroxyproline (HYP) showed higher deposition in the HPL-only and xenogeneic

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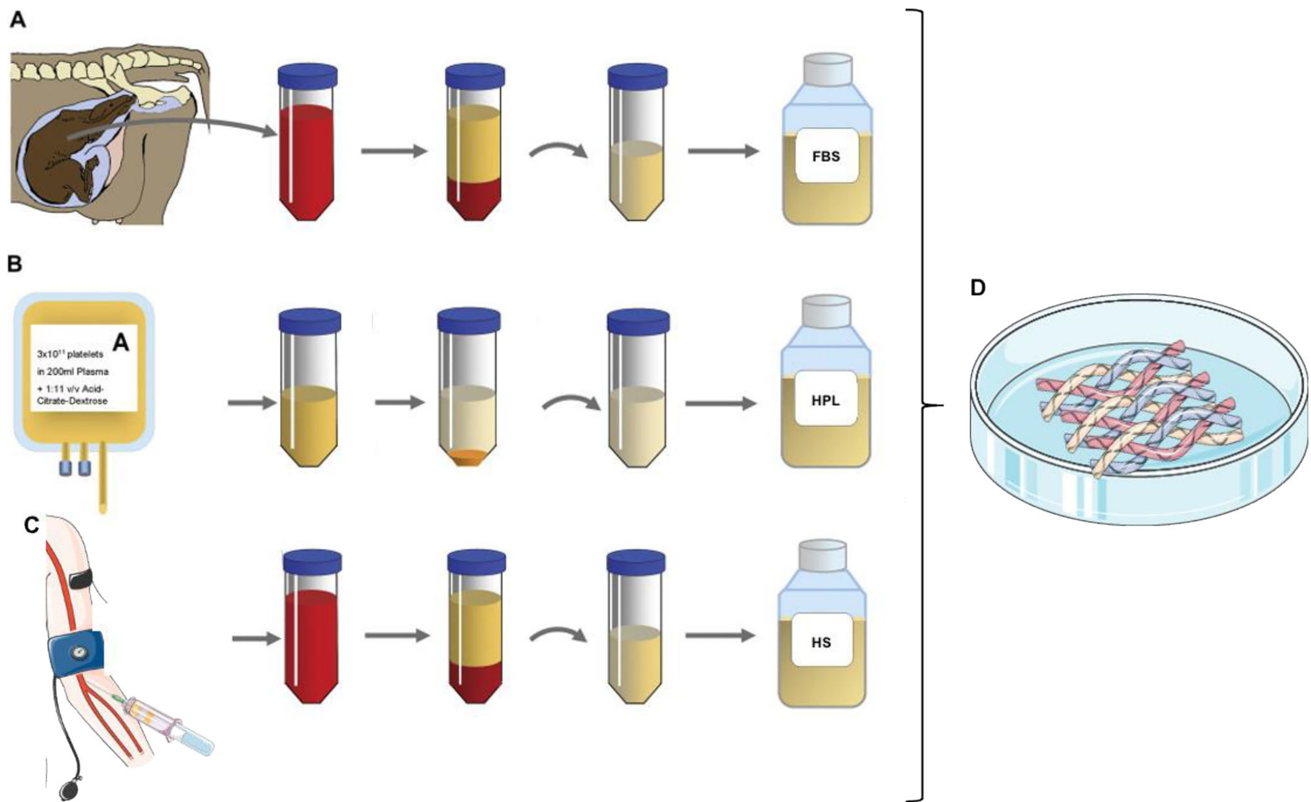

Figure 1. Schematic representing the different human tissue-engineered matrix (hTEM) culture conditions tested. A) Fetal bovine serum (FBS, control), B) human platelet lysate (HPL), and C) human serum (HS) were used as medium supplements for the development of hTEMs. D) The tested protocols were as follow: 1) advanced Dulbecco's Modified Eagle Medium (aDMEM) supplemented with 10% FBS (control), 2) aDMEM supplemented with 10% HPL (HPL-only), 3) aDMEM supplemented with 10% HPL and 2% HS (xenogeneic serum-free group).

serum-free groups compared to control samples (Figure 2E,F). Taken together, our data indicate as HPL-only may not be a reliable substitute for FBS, while medium supplemented with both, HS and HPL demonstrated to be a promising candidate showing more pronounced and organized tissue formation.

2.2. Proteins and Growth Factors Content in Culture Medium Supplements

Due to the histological differences in terms of neotissue formation observed in the hTEM, the total protein concentration of commercially available human blood serum (type AB from male clotted whole blood) and heparin-free HPL was measured with BCA assay (**Figure 3**A). Here, a higher protein content was detected for HS than HPL. While HPL is rich in growth factors, the HS from adult donors may contain lower levels of growth factors, that are required for cell proliferation and ECM formation.[19] As TGF-β1 is an important player involved in the proliferation and ECM secretion of fibroblasts, its concentration in the cell culture supplements was quantified. Results confirmed that HPL has higher levels of TGF-β1 compared to HS (Figure 3B). Metabolic activity was tested via lactate secretion of hDF seeded on scaffolds. Results showed that the secretion levels were comparable between the control and the xenogeneic serum-free groups (Figure S1, Supporting Information). Based on this notion, a xenogeneic serum-free tissue culture medium supplemented with both, HPL and HS, was used for the subsequent experiments to obtain a higher expression of collagenous fibers and a more organized ECM.

2.3. Mass Spectrometry Characterization of hTEM

In a next step, an exploratory proteomics assay was performed to assess the proteins that are present in the hTEMs, with a particular focus on the identification of potential bovine xenogeneic antigens.

The proteins in the hTEM were identified using shotgun proteomics, entailing separation by liquid chromatography and analysis by tandem mass spectrometry (LC/MS–MS). Unique peptide count showed abundance of peptides present was similar between the control and xenogeneic serum-free hTEM (**Figure 4**A). When comparing collagens, xenogeneic serum-free hTEM showed a higher level of a range of collagens produced compared to the control hTEM (Figure 4A,B). Few bovine peptides were detected in xenogeneic serum-free hTEM which could be derived from the initial composition of standard advanced DMEM (Figure 4C). Importantly, these identified bovine peptides were barely at the detection level for the tested

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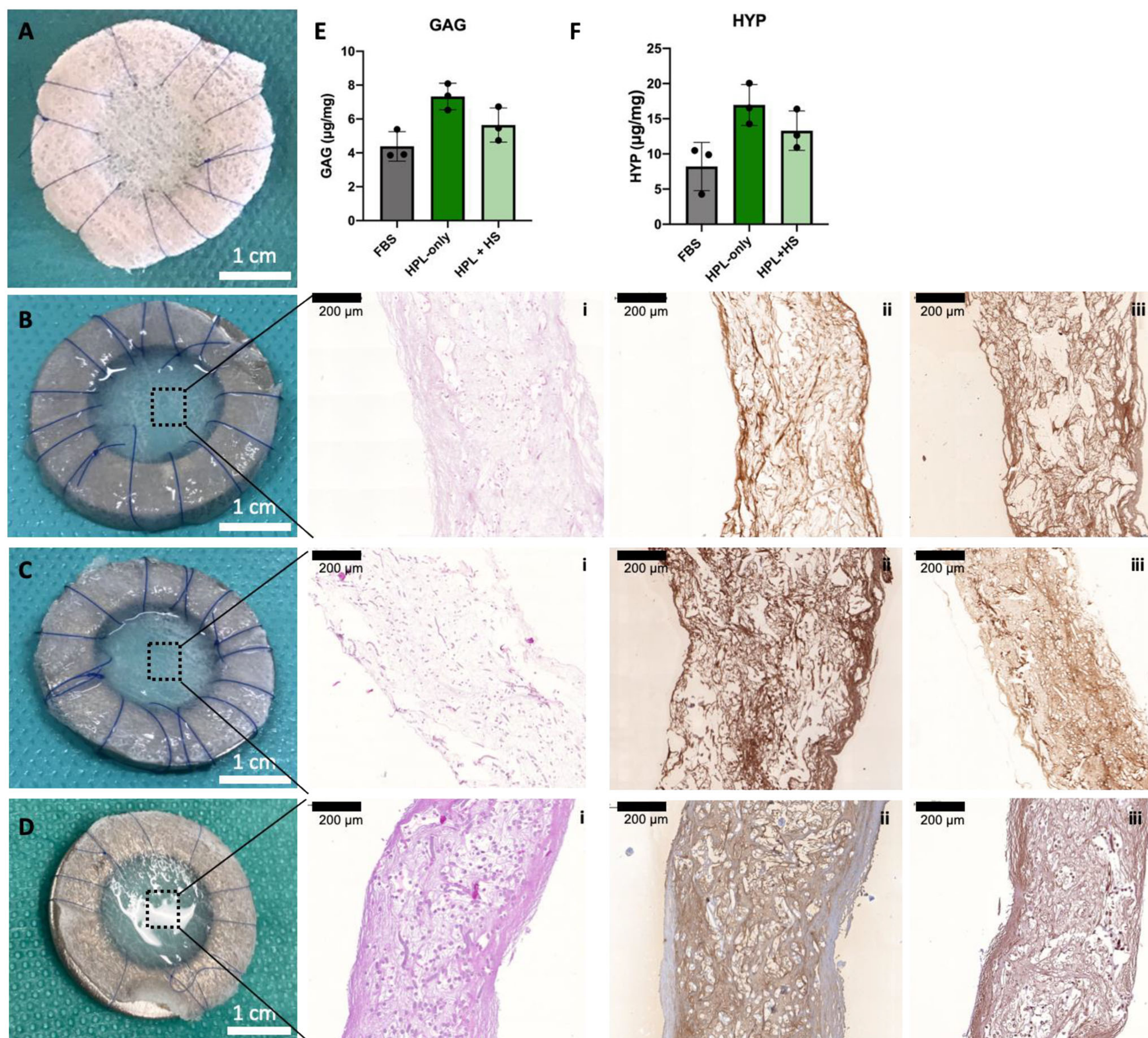

Figure 2. Qualitative and quantitative evaluation of human tissue-engineered matrix (hTEM) patches after 4 weeks culture using different protocol supplementation. A) Macroscopic image of the polyglycolic acid (PGA)/poly-4-hydroxybutyrate scaffold sutured onto a stainless steel metal ring before cell seeding. B) Representative image of a control hTEM after 4 weeks culture using fetal bovine serum (FBS) as serum supplement. i) Hematoxylin and eosin (H&E) staining shows neotissue deposition, ii) Collagen I and iii) Collagen III stainings show presence of collagen fibers. C) Representative image of a hTEM after 4 weeks culture using human platelet lysate (HPL) as serum supplement. c) H&E staining is characterized by poorly organized tissue and sparce collagen fibers as shown in ii) Collagen I and iii) Collagen III stainings. D) Representative image of a hTEM after 4 weeks culture using HPL and human serum (HS) as serum supplements. i) H&E as well as ii) Collagen I and iii) Collagen III stainings show more pronounced and organized neotissue formation for hTEM cultured with xenogeneic serum-free medium in comparison with the control and HPL-only groups. (scale bars 200 µm). E) Glycosaminoglycans (GAGs) content showed higher expression in patches cultured with HPL, comparable to levels in xenogeneic serum-free patches (n = 3). F) Hydroxyproline (HYP) content showed higher expression in patches cultured with HPL, comparable to levels in the xenogeneic serum-free patches (n = 3). Dot plot graphs present mean ± standard deviation (center line and bounds of error bars, respectively).

xenogeneic serum-free condition, and 3-fold higher for the control hTEM, which accounts for bovine proteins present in the FBS and the fibrin seeding gel (Figure 4C). This data is suggestive, that xenogeneic serum-free hTEMs contained all the essential ECM-specific and cellular peptides in comparable levels as in the control hTEM, but with only residual amount of bovine peptides.

2.4. Evaluation of hTEMs Hemo- and Biocompatibility Properties

To evaluate hTEMs hemo- and biocompatibility, we measured the levels of thrombin generation when cultured with plasma, as well as complement activation, as measured by C5 convertase.

Thrombin is a key enzyme of the blood coagulation cascade, which generation time and maximum amount give a direct input

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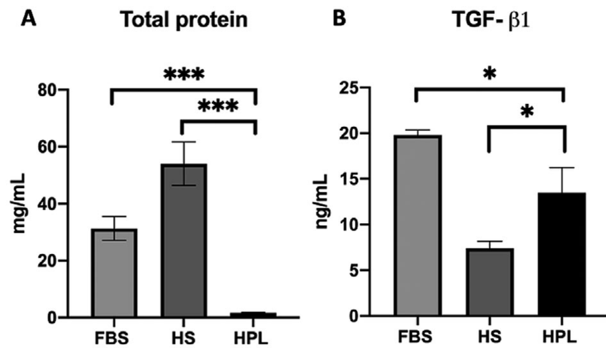

Figure 3. Characterization of protein and TGF-β1 concentration in tested medium supplements. A) BCA assay measuring total protein concentration in the supplements (n = 3). Human serum (HS) shows the higher expression of total protein content, human platelet lysate (HPL) shows significantly less than the control, and to HS group ($p < 0.001$). B) ELISA quantification of TGF-β1 concentration in the supplements show higher expression in the HPL supplement than the HS supplement ($p < 0.01$).

regarding the thrombogenicity of a biomaterial. The assessment of thrombin behavior is suited to evaluate the hemocompatibility of biomaterials and medical devices according to the international standard ISO 10993–4:2002.[20] Thrombin generation time for hTEM was found to be comparable to the reference materials, low-density polyethelene (LDPE) and polydimethylsiloxane (PDMS) (**Figure 5**A). Maximum thrombin generation levels were higher in the hTEM compared to the reference materials (Figure 5B) as exposed collagen fibers initiate the coagulation cascade thereby stimulating thrombin activation. Upon implantation, this effect is expected to occur also in vivo, until the full hTEMs endothelialization occurs. The measurement of complement convertase C5 analyzes complement activation by biomaterials. During incubation with plasma, complement factors bind to the surface of the material, which leads to the formation of the complement convertase complex. This method is suitable to evaluate bio- and hemocompatibility of biomaterials and medical devices according to the international standard ISO 10993–4. We measured the levels of complement convertase C5 in the control and xenogeneic serum-free hTEM and compared to the standards LDPE and PDMS (Figure 5C). The levels of complement convertase activation were significantly increased compared to the polymer controls. This confirms the results obtained in previously reported studies, where exposed collagen fibers of implanted biomaterials activated the complement system.[21]

2.5. Evaluation of Calcification in hTEM

To date, preclinical acute studies on hTEMs pointing to relevant calcification risks in vivo have not been reported.[7,22] Similarly, longer-term preclinical studies using tissue-engineered matrices either obtained from ovine or human cell-sources,[23–25] did not show any evidence of significant calcification risks in both, allogenic and xenogeneic animal models.

Due to the lack of knowledge related to the hTEM risk of calcification in vivo, we systematically approached in vitro testing to understand if and to what extent calcification phenomena may occur in the hTEM. Here, the control hTEM and the xenogeneic serum-free hTEM were compared using standard or osteogenic medium as culture conditions (Table S1, Supporting Information). An induced calcification assay was performed,[26] whereby valvular interstitial cells (VICs) seeded on both, control hTEMs and xenogeneic serum-free hTEMs were cultured for 21 days using standard or osteogenic medium. The presence of calcific deposits was then analyzed. For both hTEMs groups, histological analysis showed absence of calcific noduli when cultured with standard medium (**Figure 6**A,E). To the contrary, but as expected, when cultured under accelerated osteogenic conditions, spot-dependent calcific deposits were detected on hTEMs. The nodules were primarily detected on the apical side of the hTEM, regardless whether control or xenogeneic serum-free, correlating to the area of seeded VICs (Figure 6B–D,F–H). The area of the calcific nodules and the intensity of the color also appeared to be similar between both groups (Figure 6I,J). Taken together, our data indicate that VICs do not spontaneously calcify when cultured on the control or xenogeneic serum-free hTEMs under standard culture conditions, whereas when supplemented with osteogenic medium, calcification is observed in both hTEM groups with comparable characteristics as to number and intensity of calcific nodules. In addition, we analyzed the expression of calcification related genes, BMP-2 (bone morphogenetic protein 2), POSTN (periostin), and TSP-1 (thrombospondin 1), as well as myofibroblast-related gene ACTA-2 (alpha smooth muscle actin 2) (**Figure 7**). ACTA-2 did not appear to be upregulated upon stimulation with osteogenic medium, which is in agreement with previously published results, that osteogenic stimulation inhibits myofibroblastic pathway in healthy VICs (Figure 7A).[26] Furthermore, we observed that out of the analyzed markers, only the osteogenic marker BMP-2 was found to be upregulated when VICs were cultured in osteogenic medium, albeit there was no significant difference between control and xenogeneic serum-free hTEMs ($p = 0.12$), (Figure 7B). BMP-2 is an important molecule in vascular calcification, which upregulation goes in line with the current knowledge, that osteoblastic differentiation of VICs is largely orchestrated by BMPs.[27] Considering the soft material properties exerted by xenogeneic serum-free hTEMs, which are comparable to our control group (**Figure 8**), we may expect a resolution of the BMP-2 expression over-time, as BMP-2 is usually upregulated in stiffer tissues.[28]

2.6. Optimization/Development of Xenogeneic Serum-Free hTEM for High-Pressure Applications

2.6.1. Xenogeneic Serum-Free hTEMs Manufacturing and Characterization

To optimize a xenogeneic serum-free hTEM toward high pressure applications (i.e., aortic applications), the newly developed protocol was tested in tandem with a pulsatile bioreactor system. After 4 weeks culture in a pulsatile bioreactor system, hTEMs supplemented with either FBS ($n = 4$, control) or xenogeneic serum-free ($n = 4$) medium were characterized via histological assessment (Figure 8A–H). Hematoxylin and eosin (H&E) staining for the control samples showed a dense and homogeneous distribution of a collagenous ECM (Figure 8B) with abundant

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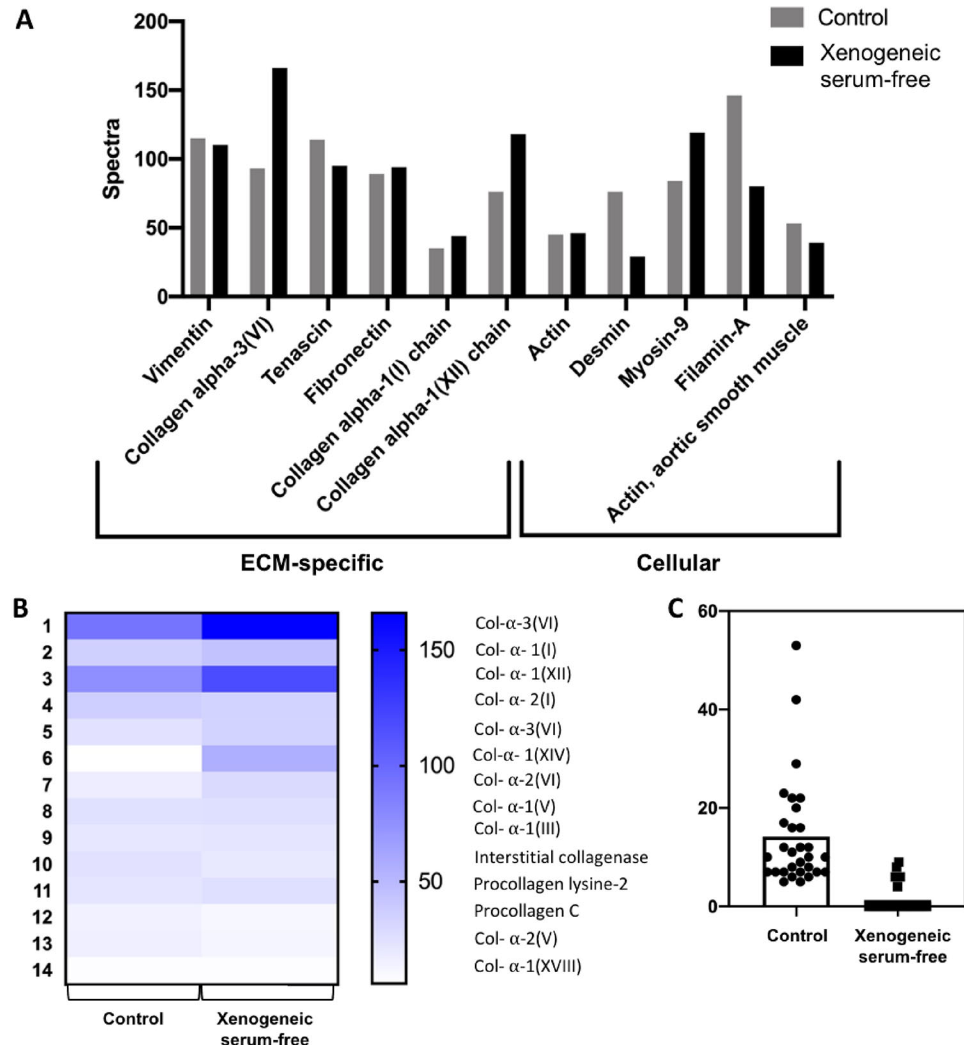

Figure 4. Liquid chromatography and analysis by tandem mass spectrometry (LC/MS–MS) of human tissue-engineered matrix (hTEM). A) The most abundant proteins detected, displayed as normalized unique spectrum count in the hTEM cultured with fetal bovine serum (FBS) supplement (control) and with xenogeneicl serum-free supplement. B) Heat map of collagens displayed as normalized unique spectrum count in the hTEM cultured as control and with xenogeneic serum-free supplement. C) LC/MS–MS of the xenogeneic peptides detected, displayed as normalized unique spectrum count in the hTEM cultured with FBS supplement (control) and with xenogeneic serum-free supplement. For spectral counting only spectra with count above 4 was considered to ensure confidence of identification.

presence of collagen I and collagen III (Figure 8C,D). Similarly, for the xenogeneic serum-free hTEMs, we observed a dense distribution of collagenous ECM (Figure 8F) with abundant presence of collagen I and collagen III (Figure 8G,H). To analyze and compare the nonlinear hTEM material properties, first the true stress and true strain were derived from the force–displacement data and two Young's moduli were fitted (Figure S2, Supporting Information). Biaxial tensile tests of the control and xenogeneic serum-free hTEM (n = 3) showed anisotropy of the material (Figure 8I,J), suggesting a consistent collagen fibers alignment in the tissue. The individual stress-stretch data for the tested samples (Figure 8I,J) demonstrated nonlinear responses. The hTEM were stretched equibiaxially in direction X (Figure 8I) and direction Y (Figure 8J). These data demonstrate there is no difference between control and xenogeneic serum-free samples in terms of mechanical properties. GAGs and HYP showed similar expression levels among the tested groups (Figure 8K,L).

2.6.2. Xenogeneic Serum-Free hTEM-Based Heart Valve Manufacturing

To facilitate the adaptation of xenogeneic serum-free hTEM culture protocol x support was designed, and 3D printed (n = 4) to prevent polymer deformation. After 4 weeks culture in a pulsatile bioreactor system, hTEMs supplemented with either FBS (n = 4) or xenogeneic serum-free (n = 4) medium were characterized. The macroscopic examination of the structure of the newly produced ECM presented a shiny and uniform neotissue deposition throughout the construct (Figure 8M–O). Further manipulation

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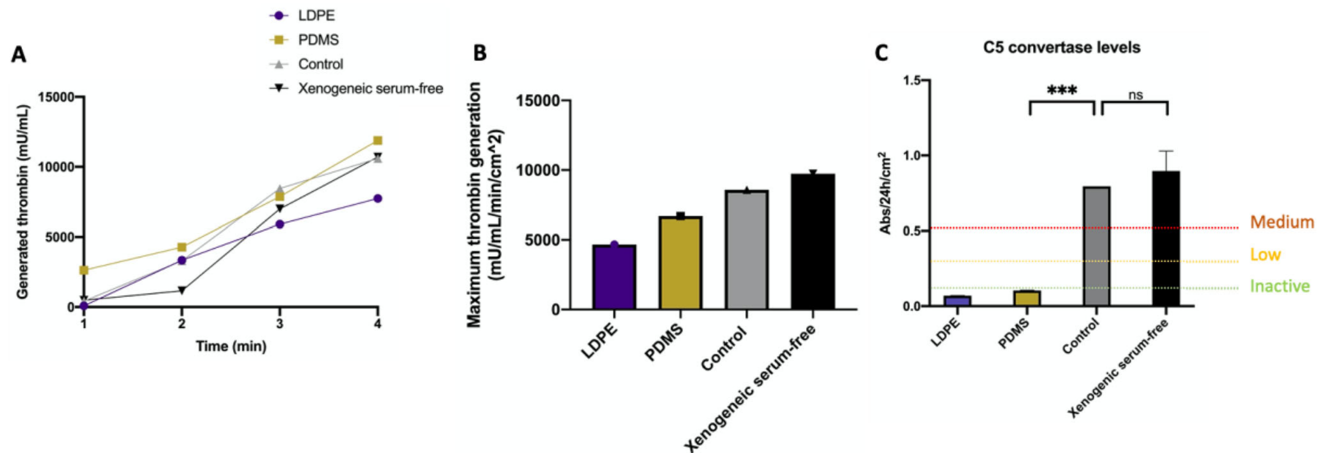

Figure 5. Hemocompatibility assessment of human tissue-engineered matrix (hTEM). Low-density polyethelene (LDPE) and polydimethylsiloxane (PDMS) were used as inactive and low reactive reference materials. A) Thrombin generation over time (n = 3). B) Maximum thrombin generation (n = 3). Maximum thrombin generation is defined as the maximum increase between the timepoints of the thrombin generation over time. C) Complement convertase levels (n = 3). One-way ANOVA performed for statistical analysis. ***p < 0.001, ns = nonsignificant).

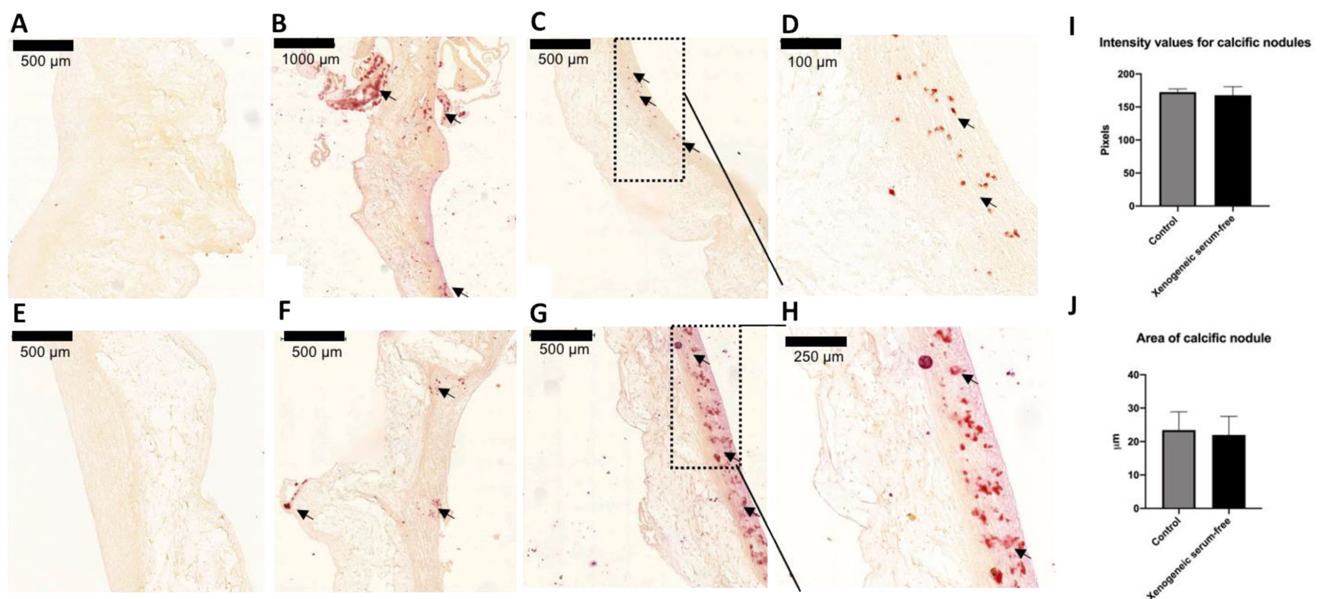

Figure 6. Histological analysis of human tissue-engineered matrices (hTEMs) cultured with valvular interstitial cells (VICs) in standard and osteogenic medium for 21 days. A) Control hTEM cultured in standard medium. B,C) Control hTEMs cultured in osteogenic medium showing calcification areas as stained by Alizarin Red (arrow heads). D) 10× magnification of a calcification loci as represented in panel C. E) Xenogeneic serum-free hTEM cultured in standard medium. F,G) Xenogeneic serum-free hTEMs cultured in osteogenic medium showing calcification areas as stained by Alizarin Red (arrow heads). H) 5× magnification image of a calcification loci as represented in panel G. I) Intensity values for calcific nodules as measured by Image J do not present any significant difference among the control and the xenogeneic serum-free groups. J) Area of calcific node in hTEMs does not present any significant difference among the control and the xenogeneic serum-free groups.

for suturing did not alter the quality of the ECM. The tested hTEMs ($n = 6$) were successfully sutured onto JenaValve stents (Figure 8M–O) and withstood the hydrodynamic valve tester for 1 h under physiologic pulmonary and aortic conditions (Figure 8P). The tissue-engineered heart valves showed complete and normal opening and closing behavior. None of the valves showed evidence of leaflet rupture or tears in the commissure points after test.

3. Discussion

FBS remains a widely implemented cell culture media supplement despite longstanding practical, clinical and ethical controversies over its use. The immunogenicity of cells cultured in FBS has raised many concerns for the therapeutic effects of cellular therapies, as seen in immune reactions reports after infusion of lymphocytes,[29] injection of human mesenchymal stem

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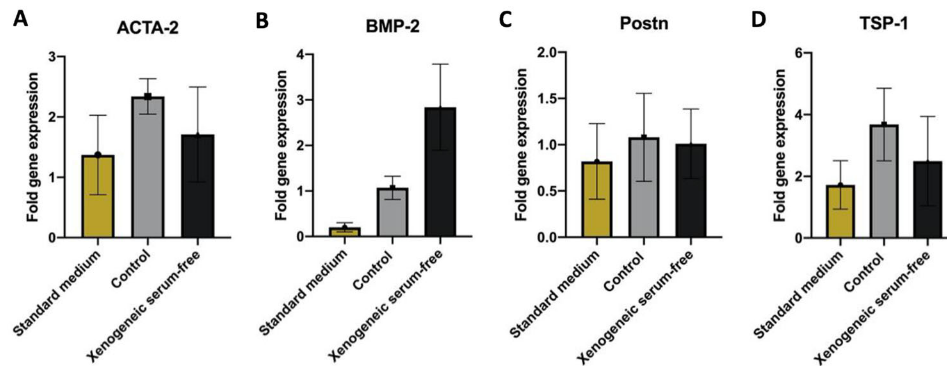

Figure 7. Gene expression profile of human tissue-engineered matrices (hTEMs) seeded with valvular interstitial cells (VICs) in standard and osteogenic medium under static conditions for 21 days. The gene expression is displayed in dCt and normalized on the average expression level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A) ACTA2 expression, B) BMP-2 expression, C) POSTN expression, and D) TSP-1 expression. The error bars depict standard error of mean (SEM). Sample size per group: n = 3.

cells,[30] skin grafts transplant of keratinocytes[31–33] and as reviewed elsewhere.[34,35] Such immunological reactions caused by medium-derived FBS proteins still represent a significant challenge for a broad and safe application of cells and tissue engineering therapies into the clinic. European regulatory bodies are currently recommending to replace FBS with material of nonanimal origin sources or to reduce its use.[17] Thus, research on substitutes for FBS in cell culture media, that allow reproducible results in terms of cell growth and proliferation, is currently ongoing.

In the present study we were able to systematically develop a novel xenogeneic serum-free hTEM culture protocol, and show the hTEM overall promising tissue formation and composition, hemo- and biocompatibility in comparison to standard tissue engineering protocols using FBS. Many efforts have been made to replace animal-derived supplements and reagents with human blood derivatives, such as human platelet lysate,[36] autologous serum[37] or pooled human AB serum.[38] HPL has been investigated as a replacement for FBS,[39] and has been shown to perform well especially in the setting of wound healing. Recently, several studies have reported reduced inflammation, improved bone regeneration and hard and soft tissue would healing, when HPL was used as a medium supplement.[40,41] In this context, we have previously shown improved pre-endothelization potential of endothelial progenitor cells on hTEMs cultured with HPL, demonstrating the capacity to form microvessel-like structures.[42] However, while some studies reported HPL alone to successfully replace FBS in tissue culture, others have shown limited cellular proliferation rates,[43] which may in clinical setting limit the expansion of cells or ECM formation.

hDFs have been shown to proliferate in the presence of HPL with shortened population doubling time compared to FBS.[44] However, fibroblasts cultured with HPL have lower expression of collagen I, collagen III, and fibronectin, as well as slower wound closure.[44] As these are important ECM protein components of vascular and valvular structures, HPL alone may not be optimal for the development of ECM-based cardiovascular constructs. Our data, further suggest that hTEMs produced with HPL only were qualitatively not robust enough, showing limited collagen formation and of heterogeneous nature. Thus, the use of HPL in hTEM culture remains controversial, whether it is able to support comparable cell growth and proliferation to FBS. In fact, while HPL is capable of activating cell proliferation machinery alone (e.g., ERK and AKT phosphorylation, Cycline D1 induction), HPL itself as a single additive is insufficient to support cell proliferation unless additional plasma or serum components are also present in the culture medium.[43]

Alternatively, the use of HS in cell culture has shown conflicting results. Several studies reported HS to be an effective supplement and clinically favorable describing promising results in terms of cell expansion and tissue formation,[45,46] maintaining expression of collagen I, collagen III, and fibronectin.[47,48] However other studies[49] showed an increased proliferation of human bone marrow mesenchymal stem cells induced by autologous serum compared to FBS, but not by allogenic serum, while a less effective differentiation of human pre-adipocytes was reported by Koellensperger et al. (2006).[50]

The combination of both, HPL and HS in the cell culture has been used previously[43,51,52] with successful results. A particular focus in our study was to assess the feasibility to implement a xenogeneic serum-free protocol for the expansion of hDF and the manufacturing of next-generation hTEMs in the context of tissue-engineered cardiovascular replacement, and specifically for heart valves. Here, we have implemented commercially available heparin-free growth factor rich HPL and adult donor AB- serum. Characterization of these two products and investigation of their biological activity let us to conclude, that HPL alone was unable to support hDF proliferation, unless serum components were present. This study is in agreement, with previously published results, using primary cultures of monkey arterial smooth muscle cells[53] and Swiss mouse 3T3 cells.[54] Both cell types requiring platelet-derived growth factors together with plasma components, to maintain cell growth, whereby platelet factors alone were not enough to promote proliferation. Thereby our study confirms previously published results, that the combination of two components–HS and HPL–was highly effective at supporting proliferation of primary mammalian cell cultures, at rates comparable to control cultures with the use of FBS.[43] In our dedicated in vitro system, the use of xenogeneic serum-free supplements showed promising results in terms of reduced immunogenicity presenting scarce if absent amount of bovine proteins, as well as maintained hemocompatibility, with no increase in thrombogenicity or complement activity for xenogeneic

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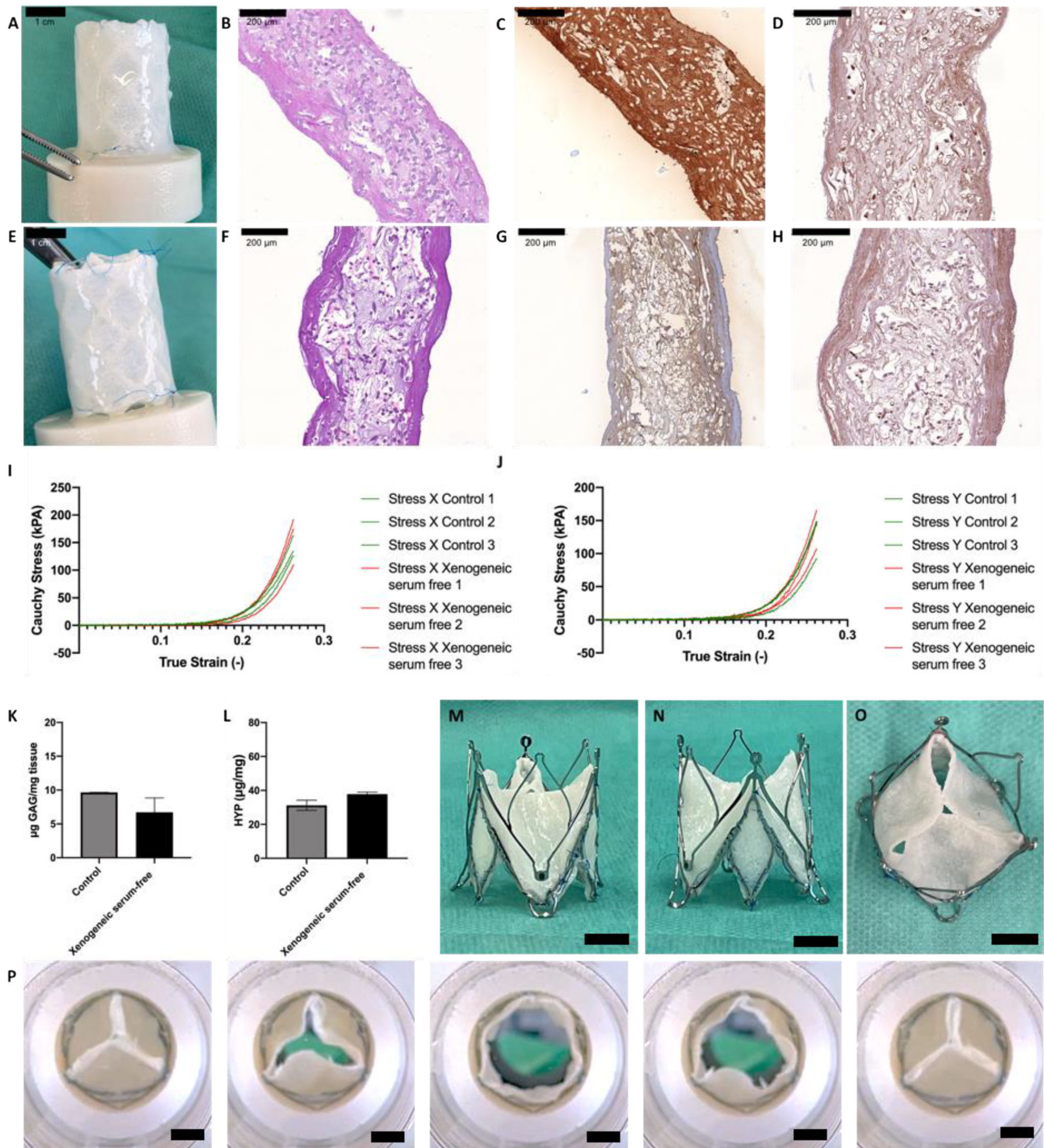

Figure 8. Qualitative and quantitative evaluation of human tissue-engineered matrices (hTEMs) after 4 weeks culture in a bioreactor system. A) Macroscopic image of a control hTEM after 4 weeks culture. B) Hematoxylin and eosin (H&E) staining shows neotissue deposition. C) Collagen I and D) Collagen III stainings show presence of organized collagen fibers. E) Representative image of an xeneogeneic serum-free hTEM-based valve. F) H&E as well as G) Collagen I and H) Collagen III stainings show more pronounced and organized neotissue formation for hTEM cultured with xenogeneic serum-free medium (scale bars 200 µm). I) Biaxial tensile testing of hTEM depicting stress versus strain in X direction. J) Biaxial tensile testing of hTEM depicting stress versus strain in Y direction. K) Biochemical characterization measuring glycosaminoglycan (GAG) and L) hydroxyproline (HYP) levels in the hTEM-based valves (n = 3). M–O) Macroscopic appearance of the decellularized tissue-engineered heart valve incorporated in the JenaValve stent (side and top view). P) Representative movie stills of the opening and closure behavior of the tissue-engineered heart valve during the cardiac cycle simulated in the pulse duplicator system at aortic conditions. Images: A, E, M–P scale bars represent 1 cm.

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serum-free hTEMs when compared to the control. As reported in our study, the presence of residual bovine peptides in the xenogeneic serum-free hTEM can be attributed at the composition of standard advanced DMEM. As albumin is one of the most abundant protein presents in serum and culture medium supplements, we could assume the detected peptides refer to residual albumin amino acids. Their scarce presence is however of low concern as native bovine albumin peptide sequence is conserved up to the 75.6% with human albumin, showing similar 3D structure.[55] Considering the conformational changes underwent by native peptides after cellular intake, including protein breakdown, the risk of such peptides to trigger an immune response to hTEMs should be low, but its thorough assessment should be integrated as a basis for a safe clinical translation. To our end, ISO conform biocompatibility tests performed with human plasma on xenogeneic serum-free hTEMs did perform similarly to control hTEMs, thereby reducing the risks of a major inflammatory response once in vivo as reported elsewhere.[56]

Calcific aortic valve disease (CAVD) remains the most prevalent valvular disorder, necessitating valve replacement.[57,58] Calcification is one of the main modes of failure in biological heart valve prostheses, which requires reoperation in 10–20 years post implantation. This is driven by tissue stiffening, and importantly by antigenic residuals following glutaraldehyde fixation of the implants. So far, the risk of calcification for hTEM-based implants remains low, even though existing evidence is limited to acute follow-ups.[7,22] A long-term study using ovine cell-based valves did not show relevant calcification in allogenic setting.[23] Similarly, a preclinical study in the xenogeneic setting showed absence of calcification, whereby engineered human acellular vessels were implanted in a baboon model.[24] The assessed low risk of calcification onset was independent from the bioengineering approach used to produce the tissue-engineered constructs. However, so far there is no long-term evidence for hTEM-based valves either implanted in the ovine model nor in humans. The main goal for hTEM-based implants is to promote adaptive in situ remodeling, while preventing chronic inflammation that could result in calcification onset.[58] However, data from calcification studies in animal models may not be relevant for translation into humans, as in the case of hTEMs will represent a xenogeneic setting. This is why the development of reliable calcification tests that could assess the material properties is of vital importance for testing and selecting novel cardiovascular implants.[59] In this study, we have induced calcification of VICs cultured in osteogenic medium on the hTEM and examined the difference in the extent of calcification between the control and xenogeneic serum-free hTEMs. VICs are the most prevalent cell type in heart valve tissue, and they actively regulate the structure and composition of the ECM during wound healing, as well as valve disease progression.[60] In our study, VICs cultured on control or xenogeneic serum-free hTEM under standard medium composition did not show the formation of calcifications, which is in line with previously published data that harvested native VICs do not calcify with standard medium.[26,61] This also indicates the rather principally low calcification risk of hTEM.

However, when cultured in osteogenic medium, as expected and as reported in literature,[26] VICs showed calcifying properties, with deposition of mineralized nodules on both, control and xenogeneic serum-free hTEMs. Nevertheless, no significant difference was observed between the two groups cultured in osteogenic medium in terms of calcific deposits detected, regardless of the manufacturing protocol used, indicating absence of certain allogenic or xenogeneic antigens that could have triggered calcific deposits in one of the two groups. To summarize, xenogeneic serum-free hTEM could represent the preferred choice when translating such technologies toward the clinical setting, however it is not yet possible to assess in humans, due to the translational gap. All in all, the promising clinical results reported by similar approaches and the low risk of calcification found, may indicate such technologies as promising from a calcification perspective. Particularly, in vivo allogenic studies that implanted ovine-based TEMs into the sheep model, and indicate low calcification[23] may suggest xenogeneic serum-free hTEMs as beneficial for the clinic. In this context, the 5-year outcomes in dialysis patients who received bioengineered human acellular vessels did not show evidence of calcification demonstrating promising prospects of low calcification risks on the long term.[56]

In addition, in vitro induced calcification studies using cocultures of VICs and valvular endothelial cells (VECs) demonstrated the inhibitory effects that VICs have on the osteogenic differentiation of VECs.[61] The presence of VICs prevented both, the osteogenic medium increase in mineralized matrix as well as the induction of osteogenic differentiation markers, thereby limiting the endothelial-to-mesenchymal transition of VECs. This study highlights the importance of VICs role in valvular homeostasis, which transient short-term activation (from quiescent state to myofibroblast-like or osteoblast-like phenotype) might be useful for the resolution of inflamed tissues. In support of this, a recent meta-analysis on tissue-engineered pulmonary valve calcification potential, reported an initial calcification surge within 1-month after implantation, which then decreased between 1 and 3 months follow-up.[62] However, when persistent activation of VICs occurs, due to insufficient valve design, material properties, or improper hemodynamical loading, maladaptive remodeling of the valve may also take place. Future studies may try to build on the current work to combine VICs and VECs and observe the potential resolution of calcific deposits. Osteogenic gene BMP-2 was found to be differentially expressed between standard and osteogenically induced hTEMs. We found that BMP-2 was not upregulated in standard medium, in agreement with previously published results that BMP-2 induced calcification relies on the presence of elevated phosphate.[63] What needs to be understood is whether the discrepancy in BMP-2 levels between VICs seeded on control and xenogeneic serum-free hTEMs is correlated with the hTEMs mechanical properties. As reported by our mechanical tests, xenogeneic serum-free hTEMs show similar soft material properties as control hTEMs, thereby potentially inducing a resolution of BMP-2 expression over time, as the implants remodel in vivo. Moreover, such preliminary results together with the previously reported hTEMs soft material properties,[23] and their ability to rapidly endothelialize,[42] may warrant further in-depth investigation and if successful may also hold promise for successful clinical translation.

Nevertheless, since aortic valve calcification is a complex phenomenon orchestrated by many factors, such as valve geometry, the overexpression of multiple pro-calcific markers as well as an elevated incidence of phosphate in the blood, we do not expect

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particular maladaptive downstream events to the expression of BMP-2 in xenogeneic serum-free hTEMs. In fact, there was no difference observed in the expression of other studied genes, either cultured in standard or osteogenic medium conditions.

Eliminating xenogeneic antigens and advancing the translation of xenogeneic serum-free matrices toward the clinics becomes especially important in the aortic setting. Graft failures due to loss of integrity, inflammation, fibrosis, and aberrant immune responses have repeatedly been observed, in particular in environments undergoing high mechanical stress or pressure. Clinical trials of decellularized xenogeneic heart valve prostheses have reported negative to fatal outcomes of Synergraft, a decellularized porcine valve, after four Ross operations in children.[64] The authors suggest that incomplete decellularization and inherent antigenicity of the scaffold were responsible. In children, Matrix P, another decellularized xenogeneic heart valve prosthesis performed worse, in a trial of 16 Ross procedures, 6 out of 16 grafts required replacement within 15 months.[65] It is evident that there is a need to elucidate the immune response to xenogeneic antigens in clinical setting. Particularly, due to the regenerative properties of hTEM-based cardiovascular constructs, it is of pivotal importance that the tissue growth and remodeling outpace the degradation of PGA/P4HB fibers and breaking down of ECM components, otherwise there might be an increased risk of graft failure. Therefore, xenogeneic serum-free hTEMs, which sustain the load of the systemic circulation, and are resistant to calcification onset could potentially decrease the risk of infection, thrombosis, and rejection, while inducing remodeling and regeneration by the host, thereby representing the next-generation solution for cardiovascular replacements.

3.1. Limitations Section

This study presents several limitations, particularly on the in vitro evaluation of xenogeneic serum-free hTEMs and their calcification potential. Future studies should further investigate xenogeneic serum-free hTEM preseeded with endothelial cells in osteogenic conditions, also taking into consideration immune cell response. This experimental setting might give more insight in the calcification mechanisms after VICs and VECs exposure to osteogenic medium, enabling a more complex simulation of the (patho)physiological phenomena usually taking place in vivo. In this context, a xenogeneic serum-free hTEM in vivo characterization has not taken place. LC–MS/MS was used as an exploratory tool to determine protein abundance by spectral counting and not as a quantitative analysis. Mechanical stresses have been shown to influence the calcification of tissues[66] and future studies could further address the mechanical loading of the control and xenogeneic serum-free hTEMs to assess the differences in stiffness and susceptibility to calcification under stress.

4. Conclusions

This study demonstrates the feasibility to use a xenogeneic serum-free hTEM culture protocol for the development of cardiovascular hTEM-based constructs. Our data suggest the effectiveness of xenogeneic serum-free protocols for the development of hTEMs of comparable quality to the control matrices, and to manufacture clinically relevant off-the-shelf available implants. HDF showed similar metabolic activity in the absence of FBS when compared to the xenogeneic serum-free protocol. Biochemical and mass spectrometry characterization showed ECM-based hTEM constructs cultured in the presence of xenogeneic-free serum sustained its characteristics as compared to control. Characterization of hemocompatibility and calcification assays of hTEMs have shown the xenogeneic serum-free potential of tissue culture. Our findings will facilitate the design of a unique and clinically relevant hTEM for high-pressure applications.

Table 1. Overview if the medium composition used in the three tested groups: control, serum-free, and xeno-free.

$n = 48$	Conditioning		
	Control	HPL-only	Xenogeneic serum-free
Medium	aDMEM	aDMEM	aDMEM
Serum supplements	10% FBS	10% HPL	10% HPL and 2% HS
Additional supplements	1% Glutamax and 1% Penicillin–Streptomycin		

5. Experimental Section

Preparation and Culture of Control and Xenogeneic Serum-Free Human Cell-Derived Tissue Engineered Matrices (hTEMs): The scaffold patches ($n = 48$) (area = 6 cm^2) were produced using nonwoven polyglycolic acid (PGA) meshes (thickness 1.0 mm, specific gravity 70 mg cm^{-3}, Cellon) and coated with 1% poly-4-hydroxybutarate (MW 1×10^6, P4HB, TEPHA Inc.) in liquid tetrahydrofuran (Sigma–Aldrich). After overnight drying, the scaffolds were sutured onto stainless steel rings (diameter 28 mm; Hasler) by using continuous sutures (Yavo, PVDF, nonresorbable, 6/0 USP), and sterilized in 70% EtOH for 30 min, rinsed in distilled UHP H_2O, followed by 30 min incubation in phosphate buffer saline (PBS, Sigma–Aldrich) supplemented with 2% amphotericin (Lonza) for 30 min and stored at 4 °C until further use.

Neonatal hDFs were purchased from CellSystems Biotechnologie GmbH and subdivided into three groups, control, HPL-only and xenogeneic serum-free (**Table 1**). hDFs were expanded in advanced Dulbecco's Modified Eagle Medium (aDMEM, Sigma–Aldrich) supplemented with either 10% FBS, 1% Glutamax (Invitrogen AG), and 1% Penicillin–Streptomycin (Invitrogen AG) for the control, or with 10% HPL, or with 10% HPL and 2% HS for the xenogeneic serum-free group. The three hDF groups were cultured in a cell incubator containing 5% CO_2 at 37 °C. Afterwards, hDFs were seeded (1.0×10^6 cells cm^{-2}) onto the PGA/P4HB scaffolds using fibrin as cell carrier[67] and placed on an orbital shaker. Tissue engineered culture medium was supplemented with L-ascorbic acid two-phosphate (0.25 mg mL^{-1}; Sigma–Aldrich) and TGF-β1 (5 ng mL; Sigma–Aldrich) and replaced every 2–3 days. After 4 weeks of culture, human cell-derived tissue engineered matrices (hTEMs) were decellularized according to the previously developed protocol[68] by using a detergent solution (PBS supplemented with 0.25% Triton X-100 (Merck), 0.25% sodium deoxycholate (Sigma–Aldrich), and 0.02% ethylenediaminetetraacetic acid (Sigma–Aldrich)) to lyse the cells. Enzymatic degradation of the nucleic remnants was achieved with Benzonase treatment (EMD Millipore) in several incubation steps (for 8 h on an orbital shaker at 37 °C) in decreasing concentrations of 100, 80, and 20 U mL^{-1} in 50 mL Tris-HCl buffer solution (pH = 8), followed by washing and storage in dPBS.

Lactic acid production was measured to assess the metabolic activity of cells in medium according to the manufacturer's instructions (Promega).

Characterization of Cell Culture Supplements: Total protein content was measured via BCA protein assay kit (Pierce BCA Protein Assay kit, Thermo Fisher) in FBS (Gibco), human platelet lysate (HPL, Zen-bio) and human

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serum (HS, Sigma–Aldrich) with ELISA assay (Sigma–Aldrich) according to the manufacturer's protocol. TGF-β1 was measured in FBS (Gibco), HPL (Zen-bio), and HS (Sigma–Aldrich) with ELISA assay (Sigma–Aldrich) according to the manufacturer's protocol. The absorbance was read at 450 nm and the amount of TGF-ß1 was extrapolated from the standard curve.

In Vitro Tissue Analyses of hTEM—Biochemical Analyses: Quantification of sulfated GAGs and HYP (as an indicator for collagen content) amounts were performed as previously described for every hTEM ($n = 36$).[21] In brief, samples were digested using a buffer solution containing 6×10^{-3} M papain (Sigma–Aldrich) at 60 °C overnight. Subsequently, we compared GAGs amount with shark cartilage chondroitin sulfate (10 mg mL^{-1}, Sigma–Aldrich) as previously described.[21] Finally, HYP amount was compared with a HYP standard solution (10 mg mL^{-1}, Sigma–Aldrich) following a previously established protocol.[69] All samples were reproduced as triplicates. We normalized all concentrations to the total dry weight of the samples.

In Vitro Tissue Analyses of hTEM—Histology and Immunohistochemistry: Representative hTEMs were analyzed qualitatively for matrix composition using histology and immunohistochemistry (Ventana Benchmark) techniques. Briefly, hTEM were fixed in 4% formalin, paraffin-embedded, and cut consecutively into 5 µm slices. The presence of cells and tissue morphology were analyzed using H&E, while the deposition of collagen was assessed with collagen-1 and collagen-3 stainings. The stained samples were imaged with brightfield microscopy (Mirax Midi Microscope, Carl Zeiss GmbH) and analyzed with Panoramic Viewer software (3DHISTECH).

In Vitro Tissue Analyses of hTEM—Mass Spectrometry: Protein identification in the hTEM was performed via LC–MS/MS analysis. The samples were digested with Lys-C and trypsin using the iST kit from PreOmics, following the manufacturer's instructions. The digest was performed for 2 h at 37 °C. The digested samples were dried and dissolved in 20 µL dd H_2O + 0.1% formic acid, and then transferred to the autosampler vials for liquid chromatography-mass spectrometry analysis. The samples were diluted 1:10 and 1 µL was injected on a nanoAcquity UPLC coupled to a Q-Exactive mass spectrometer (Thermo). The acquired mass spectrometry data were processed for identification using the Maxquant search engine (V 2.0.1.0). The spectra were searched against the following protein background database: Swissprot (all species). The protein identification results have been imported in software Scaffold (Proteome Software) for protein identification. Stringent settings have been applied in Scaffold: Protein FDR: 1%, minimum number of peptides per protein: 2, peptide FDR: 0.1%.

hTEMs Hemocompatibility—Thrombin Generation: Thrombin generation was measured using a kit (HaemoScan) according to the manufacturer's protocol. Test materials and the selected reference materials were incubated with plasma, with samples collected at $t = 1$, 2, 3, and 4 min. Afterwards the samples were analyzed by reading the optical density (OD) at 405 and 540 nm. The thrombin concentration was calculated from the calibration curve of OD405 values, in which the concentration of thrombin (mU mL^{-1}) was plotted against incubation time.

hTEMs Hemocompatibility—Complement Convertase Assay: C5 convertase was measured using a kit (HaemoScan) according to the manufacturer's protocol. Test materials were incubated with plasma, afterwards the samples were analyzed for complement convertase activity by reading the OD at 405 nm.

Cell Culture for Osteogenic Differentiation: Human VICs were purchased from Innoprot, Bizkaia, Spain. VICs were cultured in standard growth medium (aDMEM, Gibco) supplemented with 10% FBS (Invitrogen, AG), 1% Glutamax (Invitrogen AG), and 1% Penicillin–Streptomycin (Invitrogen AG) at 37 °C in 5% CO_2 until confluence of 70–80% before passaging. Cells from passages 2 to 6 were used for all experiments.

Osteogenic Differentiation: To induce osteogenic differentiation VICs were seeded on hTEM patches (25×10^3 cells per patch) in standard growth medium, and after 24 h the medium was changed to osteogenic medium: standard growth medium supplemented with 50×10^{-6} M ascorbic acid (A4544, Sigma–Aldrich), 0.1×10^{-6} M dexamethasone (D4902, Sigma–Aldrich) and 10×10^{-3} M beta-glycerophosphate (G9422, Sigma–Aldrich). For controls, VICs were cultured in standard growth medium without stimulation. The osteogenic and standard growth medium were changed twice a week for 21 days as previously reported.[26] After 21 days all cells were subjected to RNA isolation and quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis or stained by Alizarin Red to visualize and quantify calcium deposits.

Gene Expression Assay: Total RNA was isolated using Trizol reagent (15 596 026, Invitrogen) according to manufacturer's protocol and quantified by spectrophotometry (NanoDrop ND-1000 Spectrophotometer, Saveen Werner). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. Reverse transcription was performed using qScript cDNA Synthesis Kit (95047-500, Quanta BioSciences Inc.) according to the manufacturer's protocol. The cDNA was diluted 1:10 with DEPC-treated water. A master mix for every primer, composed of Fast SYBR Green (Applied Biosystems), the diluted primers (0.2×10^{-6} M) and DEPC-treated water was prepared and mixed in a 384-well plate with 2 µL of cDNA. Samples were tested in duplicate for each gene. The cycling parameters were as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, annealing at 60 °C for 15 s. Denaturation was then achieved at 95 °C for 15 s, extension and read fluorescence was done at 60 °C for 1 min. RT-qPCR was run using Power SYBR Green detection PCR Master Mix (4 367 659, Applied Biosystems, Life technologies) and ABI7900 thermal cycler (Applied Biosystems). Primer sequences are presented in Table S1 (Supporting Information). The fold change in gene expression compared to 18 S rRNA was calculated using the comparative $\Delta\Delta$CT method in Excel and the graphs were plotted in GraphPad Prism (Version 8.0).

Valve Manufacturing and Characterization: Human cell-derived tissue engineered valves were manufactured using a newly developed protocol. The scaffold (area = 28 cm^2) was based on nonwoven PGA meshes (thickness 1.0 mm, specific gravity 70 mg cm^{-3}) and coated with 1% P4HB in liquid tetrahydrofuran. A scaffold support was 3D-printed using glass-filled nylon (3D Hubs) and coated in parafilm with punctured holes for medium exchange. The PGA/P4HB scaffold was sutured in a tubular shape onto the parafilm coated 3D printed support and finally sterilized in 70% EtOH, followed by a brief washing step in UHP H_2O and 30 min incubation with dPBS supplemented with 2% amphotericin (Lonza). Neonatal hDFs (Cell-Systems Biotechnologie GmbH) were seeded (1.0×10^6 cells cm^{-2}) onto the valve scaffolds using fibrin as cell carrier[67] Tissue engineered culture medium composition varied among the two main tested groups (control and xenogeneic serum-free conditions (Table 1) and was additionally supplemented with L-ascorbic acid two-phosphate (0.25 mg mL; Sigma–Aldrich). The valves ($n = 10$) were cultured in a custom-made pulsatile flow bioreactor for 4 weeks and the medium was exchanged once a week. Following the 4 weeks culture the valves were decellularized following previously described protocol.[68]

Biochemical Analyses: Quantification of sulfated GAGs and HYP (as an indicator for collagen content) amounts were performed as previously described for every hTEM-based valve.

Histology and Immunohistochemistry: Representative hTEMs-based valves were analyzed qualitatively for matrix composition using histology and immunohistochemistry as previously explained for hTEM patches.

Mechanical Testing: Square hTEM samples of 9 × 9 mm were cut and their thickness was measured between two thin metal plates using a digital caliper. Subsequently, they were mounted in a planar biaxial mechanical tester (BioTester 5000, Cellscale) using the BioRake sample mounting system (Cellscale, tine diameter 254 um, tine spacing 0.7 mm, puncture depth 1.4 mm). Samples were attached and stretched to 30% both equibiaxially and nonequibiaxially (ratios of 30:15 and 15:30 (X:Y)). Each cycle was consisted of 15 s of loading and 15 s of unloading which were repeated five times, and the final measurement was the average of two subsequent cycles.

To analyze and compare the highly nonlinear material properties, first the true stress and true strain were derived from the force–displacement data and two Young's moduli were fitted. E_{toe} (sometimes also referred to as $E_{low\ tension}$ or $E_{elastin}$) is the linear coefficient before the transition region, where the mechanical properties are still defined mainly by the ground matrix. E_{heel} (sometimes also referred to as $E_{high\ tension}$ or $E_{collagen}$) is the linear coefficient after the transition region, where the material

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becomes significantly stiffer and the mechanical properties are influenced more significantly by the stretching of the fibers in the material.

In Vitro Valve Functionality: Control TEHVs ($n = 3$), xenogeneic serum-free ($n = 3$) were assessed for in vitro functionality in a hydrodynamic pulsatile test system (HDT-500, BDC Laboratories) in accordance with ISO5840 for 1 h. The valves placed in custom-made silicone tubes with an internal diameter of 26 mm were subjected to pulmonary and aortic conditions (rate of 70 bpm, pressure of 25 mmHg and 120 mmHg respectively) at 37 °C in PBS. Flow and pressure were measured using transonic sensors and pressure sensors and hydrodynamic data measurement was performed to extract cardiac output, regurgitation fraction, leakage volume, and closing volume, in accordance with ISO5840.

Statistical Analysis: Data in the text are represented as mean ± SD, unless stated otherwise. Biochemical characterization (HYP, GAG) was evaluated with one-way ANOVA, while supplement quantification was evaluated with nonparametric unpaired *t*-test. Prism software version 8 (GraphPad Software, Inc., San Diego, CA) was used for the analyses.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

S.P. Hoerstrup is a shareholder at Xeltis BV and LifeMatrix Technologies AG. M.Y. Emmert is a shareholder at LifeMatrix Technologies AG. The other authors have nothing to disclose.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

calcific aortic valve disease, calcification, hemocompatibility, human platelet lysate, human cell-derived tissue-engineered matrices, human serum, transcatheter tissue-engineered valve

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