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Engineered tissue vascular grafts: Are we there yet?

Joao S. Soares [a,b,e,*], Sarah K. Saunders [a], Federica Potere [c], Stefano Toldo [d,e], Antonio Abbate [d,e]

[a] Department of Mechanical and Nuclear Engineering, Virginia Commonwealth University, 401 West Main Street, PO Box 843015, Richmond, VA 23284, United States
[b] Department of Biomedical Engineering, Virginia Commonwealth University, United States
[c] Department of Chemistry, Materials, and Chemical Engineering "Giulio Natta", Politecnico di Milano, Italy
[d] Department of Internal Medicine, Virginia Commonwealth University School of Medicine, United States
[e] Pauley Heart Center, Virginia Commonwealth University, United States

A B S T R A C T

Over the last 20 years, a diverse number of different approaches have been explored in trying to produce engineered tissue vascular grafts (ETVGs). If successful, this alternative source of living vascular conduits with the ability to grow, remodel, and self-repair could revolutionize vascular surgery by relieving the limiting need for autologous grafts or providing substantial benefit and improved performance over their synthetic counterparts. However, despite tissue engineering being one of the hottest topics in biotechnology in the last three decades, it is generally acknowledged that the field's performance and its potential clinical translation have been somewhat disappointing. Pilot studies with ETVGs in animal models and preclinical human trials have been encouraging, but our understanding of the design requirements for ETVGs, how to effectively create them, and how to direct ETVG integration once implanted must be improved. This article reviews the current state-of-the-art of ETVGs with emphasis on the different manufacturing approaches explored in the past and challenges encountered and tackled, with particular focus on ETVGs that are very close to making a clinical impact and may potentially begin a new era of therapy for vascular disease.

1. Introduction

Despite almost 50 years of intensive research efforts and a global burden of cardiovascular disease with limited treatment options, we still lack viable alternative conduits relieving the supply limitation of autologous vascular grafting. Synthetic vascular grafts made of polymers (e.g., polyethylene terephthalate, patented as Dacron, or expanded polytetrafluoroethylene, ePTFE) are essential tools of vascular surgery today and have had relative success in large-caliber, high-flow and low-resistance applications providing substantial benefit to aortic or iliac grafting (Ravi and Chaikof, 2010); however, small diameter (< 6 mm) arterial grafts have not yet translated into clinical effectiveness due to thrombosis and anastomotic intimal hyperplasia (Chester, 2002). While surgeons continue to implant the well-established but less-than-optimal options of the past (Berger et al., 1972), small-diameter vascular grafts have become the *"nemesis of research and a symbol for the limitations of modern biotechnology"* (Zilla et al., 2007).

With this review, we will highlight historical and recent developments in engineered tissue vascular grafts (ETVGs) for the replacement of blood vessels. Our intention is not to provide a comprehensive overview of this ever-growing field but rather to further stimulate interest in the engineering aspects of vascular tissue engineering in general. After more than 20 years since the inception of ETVGs and even with the outstanding potential of relieving such a critical supply limitation, manufacturing methods and graft performance have only recently been close to sufficient to bring this technology near clinical translation in particular applications. Advances in ETVGs have been limited partly due to the lack of systematic understanding of the diverse biological, biophysical and biomechanical factors affecting ETVG growth & remodeling during *in vitro* incubation and after their *in vivo* implantation. The former has been coined the *"Holy Grail"* of vascular tissue engineering (Conte, 1998), or in other words, attempts at "how to engineer them?"; whereas the latter, the prediction of *in vivo* performance, requires interdisciplinary development bridging the fields of immunomodulation, tissue engineering, and mechanobiology. We will highlight progress made with their manufacturing and discuss current challenges that must be addressed before goals of more widespread development are possible. Rational use of synthetic and biological materials, their combinations, and most importantly, their interactions with the host will play a critical role in the progress of vascular tissue engineering. Functional implantation, proper three-dimensional organization, and reproducible production at clinically relevant scales are all critical problems that must be addressed (Chang and Niklason, 2017). Still, at the same time, the biological,

* Corresponding author at: Department of Mechanical and Nuclear Engineering, Virginia Commonwealth University, 401 West Main Street, PO Box 843015, Richmond, VA 23284, United States.
E-mail address: jsoares@vcu.edu (J.S. Soares).

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biochemical, and biomechanical properties of ETVGs influence the inevitable *in vivo* inflammatory foreign body response (FBR) they trigger and ultimately decide their fate (Badylak et al., 2008). ETVGs must be designed as "*tuners of the immune response*" (Li et al., 2014; Mariani et al., 2019), promoting its beneficial aspects and favoring tissue regeneration and host integration without inducing (or at least limiting) its deleterious effects such as pathological fibrosis, maladaptive remodeling, and anastomotic hyperplasia. Notwithstanding, challenges in vascular tissue engineering are broadly co-shared with other areas of regenerative medicine. Solutions to these challenges will transcend their specific application to ETVGs and will have the potential to advance significantly tissue engineering and regenerative medicine overall.

2. Unmet clinical needs

Cardiovascular diseases are one of the dominant causes of death in the western world over the last decade, tolling more than 160 deaths per 100,000 individuals per year in the US alone (~ 13% of total deaths, Kochanek et al., 2020). Coronary heart disease (CHD), generally classified as the consequence of the critical atherosclerotic narrowing of the blood vessels supplying oxygenated blood to the myocardium, is responsible for almost half of its incidence (Virani et al., 2020). Prompt restoration of myocardial tissue perfusion is pivotal for preventing heart failure in patients with CHD. Current revascularization techniques consist of either percutaneous coronary intervention (PCI) with minimally-invasive stent deployment or coronary artery bypass grafting surgery (CABG). CABG surgery remains one of the most common cardiac procedures performed worldwide, even with the continuous revolution of PCI technologies since the inception of balloon angioplasty to the third-generation drug-eluting stents (an estimated 350,000 CABG procedures were performed annually in the US alone vs. ~500,000 PCIs in 2014, Virani et al., 2020). According to US and European guidelines, CABG surgery remains crucial for patients with multivessel CHD that is too complex to be treated optimally with PCI (Head et al., 2017), patients with diabetes, or with reduced left ventricular function (Fihn et al., 2014; Neumann et al., 2019). CABG surgery frequently involves multiple bypasses, and the choice of conduits has always been a continuous debate. In contemporary practice, CABG commonly employs the patient's left internal mammary artery anastomosed to the left anterior descending artery as the best option (providing >90% 10-year patency rate), and additional stenoses bypassed with transplanted vein grafts (Fig. 1A). However, substantial variability on how and which conduits are used does exist (Head et al., 2015), and common alternatives include employing the bilateral internal mammary arteries or the radial artery. Regardless, 80% of all bypass conduits are saphenous vein grafts (SVG) because of their ease of harvesting and manipulation, the

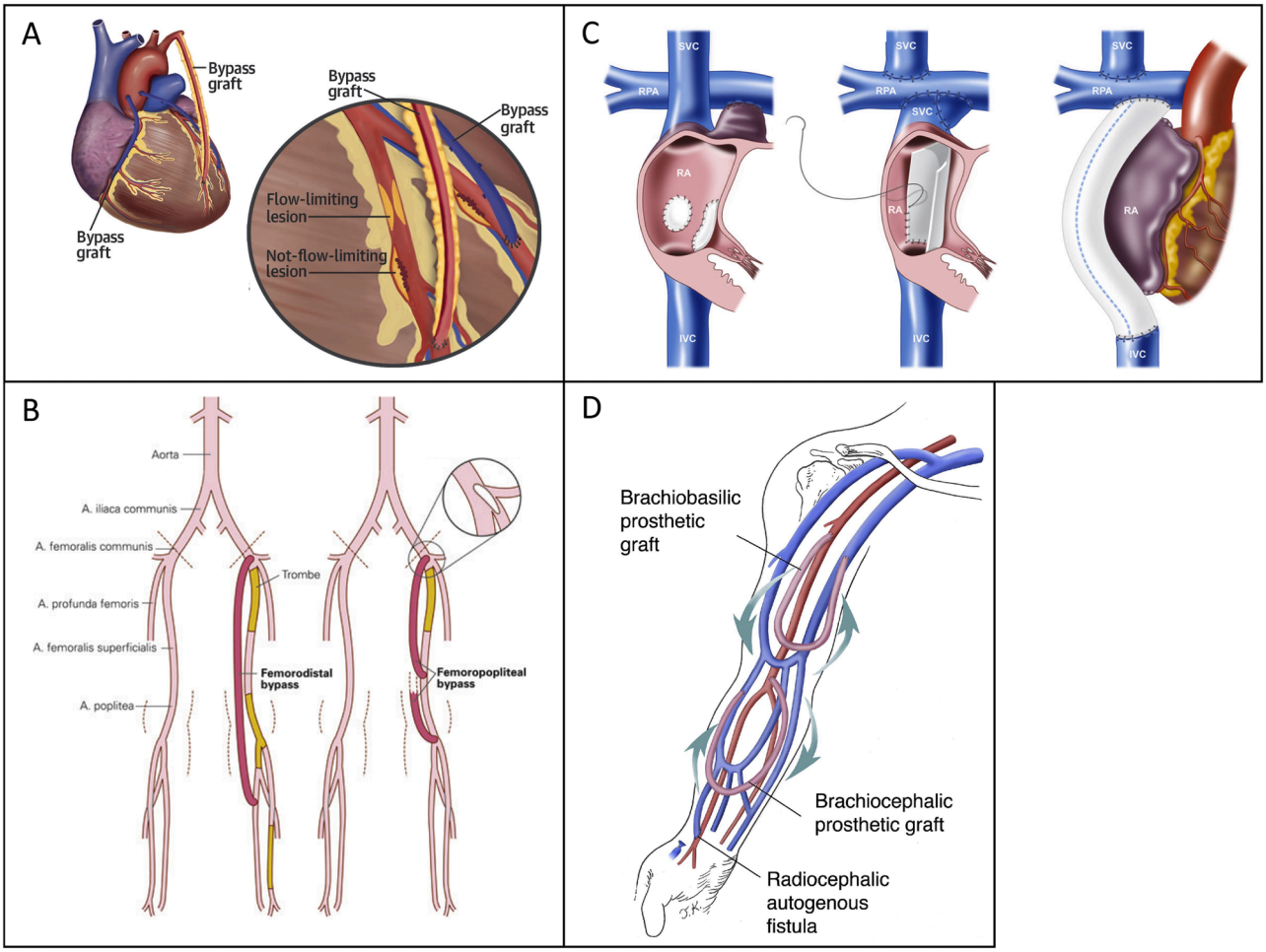

Fig. 1. Schematic illustrations of **(A)** CABG surgery showing a triple bypass made with IMA and 2 SVGs delivering blood flow beyond coronary artery blockages; **(B)** femorodistal bypass to tibioperoneal (left) and femoropopliteal bypass above or below the knee popliteal artery (right); **(C)** three Fontan procedures: classical Fontan with atriopulmonary connection, lateral tunnel with intra-atrial baffle, and extra-cardiac conduit; (D) hemodialysis assess the anatomy of the right arm showing three commonly used configurations with one autogenous radiocephalic fistula and two prosthetic brachiocephalic and brachiobasilic grafts. Adapted with permission from Doenst et al. (2019), Flørenes et al. (2009), Attard et al. (2018), Bittl (2010), Copyrights © 2019 American College of Cardiology Foundation; 2009 The Journal of the Norwegian Medical Association; 2018 Elsevier; 2010 American College of Cardiology Foundation.

lesser challenge of vein grafting (compared with multiple arterial grafting), and the often unavailability of arterial grafts due to previous usage or these being diseased themselves. However, the main disadvantage of the SVG is its tendency for progressive failure, with as many as 10-25% of SVGs occluding in the first year and as high as 50% after ten years, most often necessitating repeat revascularization (Hess et al., 2014). However, despite these problems, the majority of surgeons around the world still use SVGs in their daily CABG surgeries.

Peripheral arterial disease (PAD) is a complex and highly prevalent pathology affecting an estimated >6.5 million individuals in the US (Virani et al., 2020). PAD is highly asymptomatic, with ~40% of affected individuals not complaining of leg pain, only ~10% having the classic symptom of intermittent claudication, and just about 1% of patients having critical limb ischemia, the most severe form of PAD. However, cardiovascular and cerebrovascular disease accompanies PAD in many patients, and the mortality rate can be as high as 25% within one year (Thomas, 2007). The manifestation of PAD is highly heterogeneous, with a vast spectrum distribution, progression stages, and severity ranges. Approximately 30% of lesions are in the iliac arteries, and the remaining 70% are in the femoropopliteal and tibial track. The primary goal of any treatment of patients with PAD is either limb salvage or relief of significant lifestyle-limiting symptoms typically achieved with systemic pharmacological treatments (Norgren et al., 2007). The minimally-invasive endovascular intervention has become the first line of therapy to treat PAD and works well for shorter lesions (Conte et al., 2015). Commonly employed endovascular approaches include balloon angioplasty (plain balloon, specialized balloon, or drug-coated); bare--metal, drug-eluting, or covered stent placement; or plaque removal (atherectomy, Beckman et al., 2021). Surgery has long been considered the gold standard treatment when symptoms could not be controlled by risk factor modification, exercise therapy, or medication. Surgical reconstruction in good-risk patients, often by aortofemoral or femoropopliteal bypasses (Fig. 1B), is the standard option for longer and diffuse lesions with extensive calcification, small-caliber vessels, and poor runoff or chronic total occlusions (Pascarella and Aboul Hosn, 2018). More recently, hybrid procedures combining iliac angioplasty/stenting with femoral endarterectomy or distal bypass grafting have been increasingly employed. Autologous conduits, usually the SVG, are the preferred conduit; however, poor quality, inadequate vein size, profound vascular disease, or a history of superficial phlebitis can lead to situations where prosthetic conduits may be more appropriate (van de Weijer et al., 2015). The debate between autologous SVG and synthetical material choices for prosthetic grafts (typically either Dacron or ePTFE) has been ongoing since the inception of the surgical technique. So far, trials have not shown the superiority of one prosthetic type over another. The autologous vein conduit remains the widely preferred choice with demonstrated longer-term patency rates (fem-pop patency rates of 81% vs. 67% at two years and 69% vs. 49% at five years, Klinkert et al., 2004; infrapop patency rates of 47% vs. 30% at five years, Albers et al., 2005). The primary cause of graft failure is the result of neointimal hyperplasia and thrombus formation, causing restricted blood flow and eventual occlusion. A wide range of different targets have been the focus of research over the last decades to improve outcomes, ranging from sirolimus-eluting stent-grafts (Duda et al., 2006) or biodegradable scaffolds (Varcoe et al., 2021) for endovascular treatment, functionalization of prosthetic grafts (McAnelly et al., 2017), acellular engineered tissue conduits (Gutowski et al., 2020), and growth factors and cell therapies to promote arteriogenesis and/or angiogenesis for patients not amenable to either endovascular or surgical revascularization (Miao et al., 2014; Gu et al., 2019).

Congenital cardiovascular defects occur in about 1% of live births in the US and present themselves with significant variability in severity, ranging from minor abnormalities not requiring treatment to complex malformations that require multiple surgeries and interventions starting immediately after birth (about ~25% of the cases,Virani et al., 2020). Surgical correction aims to establish anatomic continuity and physiologic restoration and is generally reserved for patients in which other forms of therapy cannot maintain adequate circulation or the structural defects threaten the development of the heart, lungs, and other organs (Mirensky and Breuer, 2008). Congenital structural anomalies of the heart may affect any parts of the atria, ventricles, heart valves, and/or great vessels. Although primary repair of defects is sometimes possible, the implementation of prosthetic replacement grafts has allowed for the establishment of anatomic continuity and physiologic restoration in more complicated cases. Examples of interventions that require prosthetic materials are (a) the Norwood procedure temporarily connecting the subclavian artery to the pulmonary artery, typically with homograft or with synthetic graft in staged reconstruction (Ishino et al., 1999); (b) the subsequent Fontan procedure connecting the inferior vena cava to the pulmonary artery with a conduit (either external or intra-atrial (Zheng et al., 2017, Fig. 1C) or sometimes connecting the right atrium directly to the pulmonary artery with a patch (Molina et al., 1985); (c) tetralogy of Fallot may require a prosthetic conduit from the right heart to the pulmonary system and a prosthetic valve (Chiariello et al., 1975); (d) aortic valve stenosis repaired with Ross procedure replacing the aortic valve with the autologous pulmonary valve and deploying a prosthetic pulmonary valve replacement (Wiggins et al., 2021); (e) aortic coarctation with a prosthetic conduit to connect the left ventricle with the distal aorta (Tanous et al., 2009). and (f) large atrial- or ventricular-septal defects may require implantation of prosthetic patches or baffles. Although these grafts are lifesaving, they have limited durability, are prone to infection, immunologic reactivity, and thrombosis, and typically require repeat operations. Typical choices of materials for reconstruction are autologous pericardium, bovine pericardium, or synthetic materials such as knitted polyesters, Dacron, or Gore-Tex; all of these options have substantial shortcomings such as the increased risk of aneurysm formation or infection, potential for obstructive tissue ingrowth with fibrosis and calcification, difficulty to handle, and higher thromboembolic complications. Attributes for the ideal conduit are shared with conduits for CABG or PAD, such as long-term patency, availability in sizes, good handling characteristics, and low infectious and thrombogenic potential; however, such conduit does not exist. Despite clear limitations, cryopreserved aortic and pulmonary homografts are the most widely used conduits for right ventricle outflow tract reconstruction (Forbess, 2004), ePTFE and Dacron grafts for extracardiac total cavopulmonary connections (Martin et al., 2015), while pericardium (autologous or bovine) is typically employed for patch and baffle construction (IJsselhof et al., 2020).

End-stage renal disease affects nearly one million individuals in the US, with around two-thirds undergoing hemodialysis as renal replacement therapy. Durable and easily maintained vascular access in between sessions has been achieved with surgically created autologous arteriovenous fistulas (AVF) since the 1960s (Brescia et al., 1966). The properties that make AVFs the ideal vascular access for dialysis are the ability to withstand repeating venipuncture while permitting blood flow adequate for efficient dialysis (Manov et al., 2021). Autologous AVFs are the preferred form of vascular access, less prone to infection, and have higher rates of patency (Perera et al., 2004). However, some patients do not have suitable venous conduits for autologous AVF creation, and several techniques have been developed with prosthetic arteriovenous grafts (AVGs) to try to prevent the need for autologous AVFs. At AVF placement, the venous segment is transposed into the arterial circulation and subjected to increased flow and pressure (Robbin et al., 2016). Hemodynamic and mechanical parameters, specifically increased hoop and shear stresses, play an important role in regulating the natural vascular remodeling processes resulting in increased luminal diameter and wall thickness (Kheda et al., 2010). AVFs need to mature for approximately 2-3 months, and this process has a 20% to 60% failure rate (Vachharajani et al., 2021); complications frequently occur during this remodeling process, such as impaired remodeling and stenosis development due to intimal hyperplasia (Van Tricht et al., 2005),

leading to narrowing of vessel lumen, insufficient flow, and eventually thrombosis and the inability of the fistula to support dialysis (Remuzzi and Bozzetto, 2017). Most commonly, stenosis occurs in focal points where the venous outflow makes a sharp change in course. This leads to lower wall shear stress that may impede venous remodeling necessary for fistula maturation (Badero et al., 2008; Ene-Iordache and Remuzzi, 2012). Fistula dysfunction is typically diagnosed and detected with Doppler ultrasonography, directly measuring flow and providing anatomical information of venous stenoses (Bacchini et al., 2000), or with fistulography, usually along with percutaneous angioplasty the mainstay of treatment, which is very successful in changing the course of a non-maturing AVF fistula to a functioning one when performed early (Beathard et al., 2003). Notwithstanding, various treatments to salvage non-maturing AVFs have been attempted e.g., endovascular stent deployment, accessory vein obliteration, thrombectomy, systemic thrombolytics, or alternative revascularizations. Still, none showed substantial superiority vs. the highly successful angioplasty treatment (Manov et al., 2021). Although guidelines recommend "fistula first" and have encouraged an increase in AVF usage over AVGs (Allon and Lok, 2010), patients who are not candidates for AVF creation in the first place or whose AVF has failed to either mature or function will often use synthetic AVGs (Fig. 1D). Historically, AVGs were made of ePTFE, designed as a conduit and not for early or repeated puncturing, and performed poorly (Roy-Chaudhury et al., 2001). Many xenogeneic, allogeneic, and autologous alternatives to ePTFE were studied, such as bovine carotid artery (Harlander-Locke et al., 2014; Kennealey et al., 2011), bovine depopulated ureters (Das et al., 2011), bovine mesenteric vein (Hatzibaloglou et al., 2018), cryopreserved human femoral vein (Matsuura et al., 2000), or iliac artery (Ha et al., 2016). More recently, new design technologies with multilayered synthetic materials have emerged, allowing grafts to be cannulated early after placement without the need for maturation time, and were evaluated in studies with limited numbers (Al Shakarchi et al., 2015). However, none of these alternative options has performed convincingly better than ePTFE grafts (Vachharajani et al., 2021; Lawson et al., 2016).

3. Tissue engineering blood vessels

The classical paradigm of tissue engineering aims to create materials that integrate with native tissues to restore, maintain, or improve physiological function with three interdependent components - cells, scaffolds, and signals – that are essential to each other when attempting to form organized neo-tissue (Vacanti and Langer, 1999). Tissue engineering constructs, being naturally autologous, are theoretically thrombo-resistant, less prone to infection, and have growth capacity. Although some initial experimentation existed dating back to the work of Sparks in 1969, achieving tubes composed of de novo tissue generated *in vivo* through a foreign body reaction around an implanted mandrel (Sparks, 1969), the first truly tissue engineering blood vessel constructed *in vitro* was reported by Weinberg and Bell in 1986 – it was a tri-layered tube capable of withstanding physiological pressures in a mock circulatory loop with a confluent monolayer of endothelial cells, a middle layer with a high density of smooth muscle cells and extracellular matrix material, and an outer layer with adventitial fibroblasts and more matrix material (Weinberg and Bell, 1986). It was constructed with a stepped approach, first casting collagen gels with SMCs in an annular mold for a week, then with a surrounding Dacron mesh sleeve and casting the outer adventitial layer with fibroblasts and collagen for two additional weeks, and lastly, lining the lumen with endothelial cells in rotating culture for one more week. The graft grossly resembled a muscular artery, except for the Dacron mesh and subpar collagen and cell densities, but possessed other very appealing aspects such as well-differentiated SMCs appearing to secrete collagen, fully endothelialized and functional lumen producing von Willebrand factor, prostacyclin, and forming a permeability barrier to large molecules. Weinberg and Bell tested different manufacturing formulations, such as initial cell density, culture time, and collagen gel concentrations, and observed a strong relationship between constituents and mechanical performance. The Dacron mesh was crucial for the grafts to withstand any pressure; without it, the grafts were highly distensible and ruptured with <10 mmHg. However, only 180 mmHg burst strength was achieved (substantially less than the 2000 mmHg and 3000 mmHg of the human SV or IMA, respectively, Konig et al., 2009).

Due to the overwhelming lack of stiffness of the engineered tissues achieved at the time, the logical step was to embrace the ongoing synthetic graft option, leveraging their mechanical strength in conjunction with the ability of endothelial cells to modulate the vascular response to injury (Song et al., 2018). Mansfield et al. in 1975 first reported the prevention of pannus ingrowth through pre-endothelialization of synthetic grafts in calves (Mansfield et al., 1975). Subsequently, Herring et al. in 1979 investigated EC-lined synthetic grafts with diverse material and microstructural designs implanted in dogs attempting to understand the relationships between graft porosity and the benefits of single-staged seeding of ECs in host response (Herring et al., 1979). Since then, multiple studies have attempted to harness the benefits of introducing a luminal interface with enhanced biological function at implantation (Pennell et al., 1986; Noishiki et al., 1992; Zilla et al., 1994; Williams and Wick, 2004). However, EC-seeding failed to achieve any improvement when carried into clinical trials in femoropopliteal bypass grafts in the 1990s (Herring et al., 1994). Seeding densities attempted were too low, and in addition, ECs are typically challenging to harvest from patients who present the need for grafting and generally add one additional layer of technical challenges to graft development and handling. Regardless, single-staged seeding has still carried over into present-time state-of-the-art ETVG research using bone marrow as the cell source (Watanabe et al., 2001; Matsumura et al., 2003).

The ability of ETVGs to withstand physiologic pressures without employing a synthetic mesh/scaffold remained unsolved until L'Heureux et al. (1998) reported the construction of a completely biodegradable ETVG in 1998 with a cell-sheet technique that achieves burst strength up to 3500 mmHg (Konig et al., 2009). These ETVGs were constructed with two pre-cultured sheet layers, composed of smooth muscle cells (media) wrapped with the fibroblast sheet (adventitia), and subsequently endothelialized for a non-thrombotic luminal surface that strongly inhibited platelet adhesion *in vitro*. After maturation, the graft featured numerous ECM proteins such as elastin, expressed desmin by SMCs and von Willebrand factor by ECs, and demonstrated good handling and suturability in short-term grafting in a canine model but showed limited patency after seven days. Niklason et al. (1999) reported a bioreactor-mediated approach to produce ETVGs with arbitrary lengths composed of a biodegradable polyglycolic acid scaffold seeded with bovine aortic SMCs and cultured *in vitro* under pulsatile radial stretching up to 8 weeks. The resulting grafts demonstrated robust growth of engineered tissue composed of up to 50% dry weight of collagen, supporting the PGA fragments and allowing ECs to be seeded onto the luminal surface. SMCs expressed α-smooth muscle actin (SMA), calponin, and myosin heavy chain, which resembled native bovine and porcine vessels excised from young animals. These grafts possessed rupture strength of up to 2000 mmHg, which allowed the initial implantation in the saphenous artery in a swine model and remained patent for up to 4 weeks. Over the years, the same group has consistently refined and improved their technology with approaches that involved the creation of engineered collagenous ECM accreted in a fast-degrading PGA scaffold, which then is decellularized and serves as a natural scaffold for the next tissue engineering step – either re-seeded and cultured *in vitro*, or implanted directly as an off-the-shelf acellular graft that relies on host recruitment (Dahl et al., 2011).

Despite these overwhelming positive and successful initial results more than 30 years ago, maybe hundreds of different ETVG designs and manufacturing methods have been developed and evaluated *in vitro* and *in vivo*; yet, very few have progressed towards translational applicability. So far, only four ETVGs were or are currently undergoing clinical

trials – the use of ETVGs in the venous circulation as cavopulmonary conduits in children with congenital heart disease by Shinoka, Hibino, Breuer and co-workers (Matsumura et al., 2003; Shinoka et al., 2005; Hibino et al., 2010), as arteriovenous shunts for hemodialysis by Niklason and co-workers (Lawson et al., 2016) and by L'Heureux and co-workers (McAllister et al., 2009); and in above-knee femoral-to-popliteal bypass conduits in reconstructive treatment of peripheral arterial disease (for total occlusion of the superficial femoral artery when not amenable to endovascular therapy, Gutowski et al., 2020).

4. The ideal engineered tissue vascular graft

In general, the ideal ETVG would have the following broad characteristics (Walpoth and Bowlin, 2005; Pashneh-Tala et al., 2016):

(1) serve as a blood conduit with minimal flow resistance or pressure drops;
(2) withstand blood pressure while not presenting a compliance mismatch at the anastomotic site,
(3) sufficient suture retention strength and ease of surgical handling with enough flexibility with kink resistance,
(4) acceptable fatigue properties for post-implant durability without aneurysmal formation;
(5) naturally biocompatible, being non-toxic, non-thrombogenic, and resistant to infection;
(6) resistant to leakage but with adequate porosity to facilitate healing/regeneration;
(7) not promote an immunogenic response such as chronic inflammation, complement cascade initiation, or activation of the adaptive immune system while triggering an appropriate remodeling response, fully integrating with the patient, and possessing the natural mechanisms of growth, remodeling, and self-repair;
(8) easily manufactured and mass-produced with low costs, shipped, stored, sterilized, and available off-the-shelf for implantation in a wide variety of sizes (i.e., lengths, diameters, and tapering ratios)

Unfortunately, no conduit to date possesses all of these qualities and attributes. Methods to achieve them have focused on tailoring its three main components: the scaffold (biological or prosthetic), the matrix, and the endothelial cell lining.

5. The pursuit of a functional lumen

The most striking difference between an acellular prosthetic vascular graft composed of synthetic biomaterials and an ETVG is the presence of a living functional layer of ECs resting on a metabolically active SMC-rich collagenous layer. It is well established that intercellular communication between ECs and SMCs occurs since the morphogenesis of native blood vessels and regulates mural cell growth and behavior – mitogenic signals are regulated and diminished by the endothelium, and SMCs typically remain quiescent and refractory to fibroblast growth factors and platelet-derived growth factors (Dodge et al., 1993). This homeostasis has always been the driver for the pursuit of luminal endothelialization of vascular grafts, either synthetic or tissue engineered, to decrease the likelihood of SMC hyperplasia and restenosis after implantation. Once implanted, there are only three hypotheses for the possible sources for potential endothelialization (Fig. 2): (a) trans-anastomotic pannus ingrowth from the native artery itself, (b) the "fall out" endothelialization by circulating progenitor cell precursors in the blood, and (c) transmural capillarization and tissue ingrowth (Baguneid et al., 2006). The focus of earlier research was trans-anastomotic endothelialization, which showed promising results in young animal models. Still, it was seldom achieved in humans (usually elderly and diabetic) exceeding at most 10 mm inwards from the anastomosis with large portions of grafts remaining bare even after years

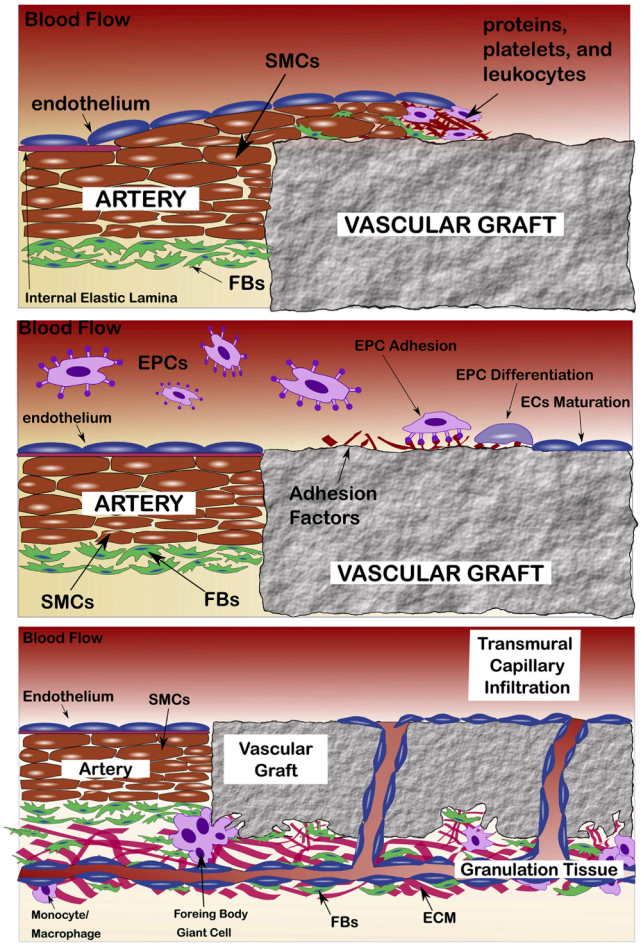

Fig. 2. Representation of vascular graft endothelialization through trans-anastomotic pannus ingrowth (top), "fall out" endothelialization from circulating cells (middle), and transmural capillarization (bottom). ECM: extracellular matrix, FB: fibroblasts, SMC: smooth muscle cell; EPC: endothelial progenitor cell. Adapted with permission from Sánchez et al. (2018), Copyright © 2018 John Wiley & Sons, Ltd.

of implantation (Zilla et al., 2007). "Fallout" healing has been identified and hypothesized as a possible source for endothelialization (Shi et al., 1994), but the evaluation of its specific contribution is challenging due to the requirement of experimental models that prevent the other mechanisms or employ specific labels for tracking blood circulating ECs (Sánchez et al., 2018). The latter, transmural capillarization, is currently the most-accepted source but probably the least well-understood. Different studies have observed capillary development into the graft wall and endothelium islands forming from the transmural capillaries (Florey et al., 1962; De Wu et al., 1996). This angiogenic process is associated with the inflammatory FBR in the perivascular space and modulated by graft properties such as porosity, pore size, and permeability. These physical properties have been critical variables in graft and scaffold design since the early days of vascular graft research (Melchiorri et al., 2013). Historically, the different contributions towards possible sources of *in situ* endothelialization are still the subject of debate and difficult to dissociate. In addition, due to the complexity of the biology of the processes, a comprehensive understanding of graft function after implantation remains elusive.

Ultimately, and because the endothelialization process is a key contributor to the success of any vascular graft, i.e., the lack of a healthy endothelial layer has been directly related to failure, most tissue engineering approaches solve this possible problem by seeding ECs or endothelial progenitor cells directly onto the surface of the grafts before

implantation (Sánchez et al., 2018). ETVG endothelialization by seeding a confluent monolayer of autologous ECs onto the luminal surface was proposed since the inception of the ETVG technology (L'Heureux et al., 1998; Niklason et al., 1999) and employed previous knowledge developed for synthetic ePTFE or Dacron grafts (Herring et al., 1979; Deutsch et al., 1998). Initially, seeding was achieved with static methods pipetting the ECs directly onto the scaffold's lumen. Static seeding was typically followed by *in vitro* culture in bioreactor systems that attempt to mimic some of the hemodynamic aspects to prepare ETVGs for *in vivo* function, reduce its potential thrombogenicity when exposed to blood, and improve neo-tissue formation and graft patency. However, this technique was limited by its poor efficiency, waiting time needed for cell adhesion, and possible cell detachment when exposed to flow. Some studies reported cell loss of 70% after a few minutes and 95% in the first 24 h after implantation (Sánchez et al., 2018).

Different cell types/sources have been used, with autologous vascular ECs isolated from peripheral blood vessel biopsies being the most common choice. The main advantage of using autologous cells is the non-requirement for immunosuppressive therapies; however, challenges to their extraction due to low quality and availability and low proliferation rates related to age and health of the donor limited their applicability. Alternatively, advances in stem cells have expanded the potential cell sources for *in vitro* endothelialization. Possible options include adult vascular progenitor cells, bone marrow mononuclear cells, stem cells from various sources (e.g. mesenchymal, adipose tissue, muscle-derived), and induced pluripotent stem cells (Pashneh-Tala et al., 2016; Herberts et al., 2011). Theoretically, these cells have the potential to differentiate into mature ECs and proliferative SMCs and form intimal and medial layers; however, harvesting these is challenging, and this technology still suffers from many technologic burdens and regulatory unknowns. Still, promising results have been obtained in particular with bone marrow mononuclear cells and endothelial progenitor cells (by Hibino, Shinoka, Breuer and co-workers), which can differentiate into EC-like phenotypes and express various EC membrane markers such as CD21, VEGFR-2, and mediate nitric oxide secretion (Melchiorri et al., 2013; Briasoulis et al., 2011). Lastly, the possibility of using non-autologous cells in vascular tissue engineering could relieve the problems associated with the quality, quantity, and timing of availability of cells and ETVGs – for example, L'Heureux and colleagues have obtained negligible immune response with allogeneic fibroblast-derived ETVGs (McAllister et al., 2009; L'Heureux et al., 2006; Wystrychowski et al., 2014), and allogeneic dermal fibroblasts have been approved and used for skin grafting without immunological issues (Pashneh-Tala et al., 2016).

Many techniques to improve seeding efficiency and patency outcomes have been developed over the years, such as formulations of culture medium, single-stage vs. multi-stage seeding, or the inclusion of mechanical/hemodynamic stresses during culture, either static or dynamic. In general, the application of shear stress and circumferential dynamic forces *in vitro* in a flow chamber/bioreactor increases seeding efficiency, enhances adhesion, ECM deposition, EC alignment, and stimulates metabolic activity such as the production of endothelial nitric oxide synthase and the expression of EC markers CD31, thrombomodulin, and von Willebrand factor (Hoenig et al., 2006; Schmidt et al., 2006). Single-stage seeding relied on harvesting cells from tissue biopsies and seeding immediately, but concerns about its application to humans due to low cell densities available to harvest and long-term maturations necessary for complete luminal lining prompted the development of the two-stage seeding process. In this process, extracted ECs are expanded firstly *in vitro* for periods up to 4 weeks such that higher cell densities can then be delivered to the ETVG to provide a more stable and mature endothelium. Additionally, multi-stage seeding can deliver multi-cell cocktails such as ECs and SMCs, or create an SMC sheet similar to natural blood vessels. Even though *in vitro* endothelization is the most common technique to achieve the desired non-thrombotic blood interface, it may not be the most effective. Reports of successful and fully endothelialized grafts suggest that the seeded cells were not detectable within a few days after implantation and might not even differentiate and populate the lumen as hoped but instead recruit host cells via paracrine mechanisms (Hibino et al., 2011a). Indeed, the possibility of generating ETVGs without cells has been explored by several research groups (yet so far with limited success, Wissing et al., 2017); however, removing cells from the ETVG equation has substantial practical and economic implications, greatly simplifying the pathway to clinical adoption and decreasing costs (Pashneh-Tala et al., 2016).

6. Options for scaffolding

Biomaterials employed as vascular grafts are diverse in form and function, and can be classified within three major areas: (1) biopolymers, in the form of either naturally occurring or manufactured/assembled scaffolds, (2) scaffolds made with synthetic polymers, either degradable or non-degradable, or lastly (3) coatings or chemically modification of these two options. Each can then proceed into the different tissue engineering strategies, e.g. implanted without cells relying on *in situ* regeneration (as currently done in clinical practice with synthetic grafts) or seeded and cultured *in vitro* to form a precursor of a blood vessel substitute in the tissue engineering approach. Many different materials for constructing vascular grafts have been explored, and some of them have entered the translational stage with animal models to evaluate post-implanation graft remodeling and long-term patency (Liu et al., 2018).

Synthetic nondegradable polymers have been widely applied as vascular grafts due to their off-the-shelf availability in various sizes and the ease with which their mechanical properties can be modified. The existing prosthetic options are currently limited to expanded polytetrafluoroethylene (ePTFE/Teflon/Gore-Tex), polyethylene terephthalate (PET/Dacron), and polyurethanes (Kannan et al., 2005). Gore-Tex is frequently used in femoropopliteal bypasses in the absence of available autologous vessels, whereas Dacron is more often used for large caliber aortic replacements (Liu et al., 2018). Both have already been in clinical application for over 50 years with extensive literature documenting clinical outcomes – Dacron as grafting material was first reported in 1958 by De Bakey and coworkers bypassing the aorta and in the peripheral vasculature in humans (De Bakey et al., 1958), whereas ePTFE was introduced by Soyer and colleagues in 1972 as venous grafts in the portal vein and vena cava of dogs (Soyer et al., 1972). Polyurethanes have been available as hemodialysis access grafts since 2000 (Glickman et al., 2001), and have been attempted in the aortoiliac position in dogs (Seifalian et al., 2003). While these prosthetic options are widely available and have a satisfactory function in large-diameter grafting in peripheral artery disease, they fail to demonstrate comparable long-term patency to autologous grafts in small diameter settings ($<$ 6mm) due to their susceptibility to thrombosis, inflammation, intimal hyperplasia, and compliance mismatch with the host vessels.

As possible viable alternatives to synthetic biostable polymers, degradable polymers have been widely explored as candidates for vascular graft materials due to the possibility of allowing for a seamless transition from a synthetic to an endogenous graft and being replaced by de novo tissue produced either by seeded cells and incubated a priori (in the traditional tissue engineering approach), or simply relying on recruited cells *in situ* from the host. Widely used materials include polyglycolic acid (PGA), polylactic acid (PLA), polycaprolactone (PCL), and many others. Different formulations yield the ability to tune their mechanical properties and degradation rates. The success of large diameter grafts with these polymers has been very reproducible in animal models in the venous circulation as IVC interpositions, showing endothelialization with ECM and SMC deposition similar to native vessels and the absence of aneurysms, thrombosis, or calcification (Liu et al., 2018). However, only limited success has been achieved in small diameters in arterial positions.

Earlier alternatives to these synthetic biomaterials (stable or

biodegradable) included the use of fresh (cold-stored) or cryopreserved homografts (i.e., allografts from cadaver donors), but these were abandoned in the early 1960s as the synthetic option became readily available and due to the inherent difficulties of preserving them, late graft deterioration, and aneurysm formation. However, they have been reintroduced over the years in carefully selected indications, e.g., for managing aortic graft infection, lower extremity primary revascularization, and simultaneous or sequential revascularization surgery in solid organ transplant recipients (Chlupác et al., 2009). The use of heterografts (i.e., xenografts from tissue from different species) and decellularized tissue scaffolds emerged in the 1980s with the overall goal of relieving the limited supply of autologous grafts and many preclinical implantations have been conducted in large animal models and human trials. Decellularized natural tissue, either of allogeneic or xenogeneic origin, allows for the preservation of the 3d-tissue architecture, which in turn, could facilitate host-cell migration with the provision of adhesion molecules and confers the ability to adapt and "grow" with the host. Examples of such tissues are the small intestinal submucosa (Lantz et al., 1992), diverse arterial or venous tissues themselves (Wissing et al., 2017), e.g., carotid, abdominal aorta, pulmonary trunks, or even biological scaffolds "synthetically" obtained from dermal fibroblasts in fibrin gel molds. Many additional biomaterials of natural sources have been employed as ingredients of manufacturing processes, such as chitosan- (Zhang et al., 2006), hyaluronan-, silk fibroin- (Soffer et al., 2008; Wang et al., 2009), collagen- (Marelli et al., 2012; Lu et al., 2013), or fibrin-based biomaterials (Huynh and Tranquillo, 2010; Syedain et al., 2011), either in the form of gels or achieved with specific manufacturing techniques such as casting or compaction. These natural biomaterials can be easily obtainable/available and are highly biocompatible. These can lead to grafts bearing a remarkable resemblance to native arteries (if successful) and can smooth the transition from implanted to the remodeled ETVG. However, the lack of stability, mechanical integrity, controllability of degradation rates, and difficult translation to clinical practice limits their development and widespread application.

7. Methods of manufacture

The creation of ETVGs can be categorized into three main methods (Fig. 3): (1) scaffold-based approaches relying on seeded cells onto either synthetic, natural, or hybrid biomaterials, (2) self-assembled vascular grafts either based on rolling cell-sheets or molding/casting cell-laden gels, and (3) acellular ETVGs form xenogeneic sources that rely solely on *in situ* cell recruitment and tissue formation/regeneration post-implantation. Methods of manufacture were extensively reviewed in general several times recently (Chang and Niklason, 2017; Pashneh-Tala et al., 2016; Carrabba and Madeddu, 2018; Park et al., 2004; Matsuzaki et al., 2019; Wang et al., 2019). For the remaining of this review, we focus on individual efforts by some research groups bringing ETVGs closer to clinical reality.

7.1. Scaffold-based ETVGs

The scaffold-based approach is certainly the most common and diffuse strategy. Physical support, which can be tailored a priori, enables seeded cells to follow the desired path during their proliferative process *in vitro* under controllable conditions inside bioreactors. However, the required technology and extended period of culture/manufacture constitute a significant obstacle to clinical translation (Carrabba and Madeddu, 2018). Niklason et al., 1999 pioneered the development of the scaffold-based methodology. Niklason and Langer (1997) first studied bovine aortic SMCs and ECs seeded onto PGA meshes and PLGA films grown under static conditions for up to 4 weeks and obtained a dense, albeit disorganized, tissue-like cell structure; however, tubular PGA scaffolds did not exhibit the physical strength required for a vascular conduit . Seminal *in vitro* experiments in the field of mechanobiology of vascular smooth muscle supported the scientific premise for mechanically-conditioned tissue engineering (Kanda and Matsuda, 1994; Birukov et al., 1995), and subsequently, the authors investigated the usefulness of applying physical forces, specifically pulsatile stretches with intraluminal pressure and flow, through a bioreactor system that attempted to mimic the *in vivo* condition during morphogenesis (Fig. 4 A, Niklason and Langer, 1997). At around the same time, Shinoka and colleagues were performing the first human implantations of ETVGs in congenital heart disease reconstructive surgery with large-diameter grafts based on scaffolds made of PGA and a copolymer of lactide and ε-caprolactone, seeded with the patient's bone marrow-derived stem

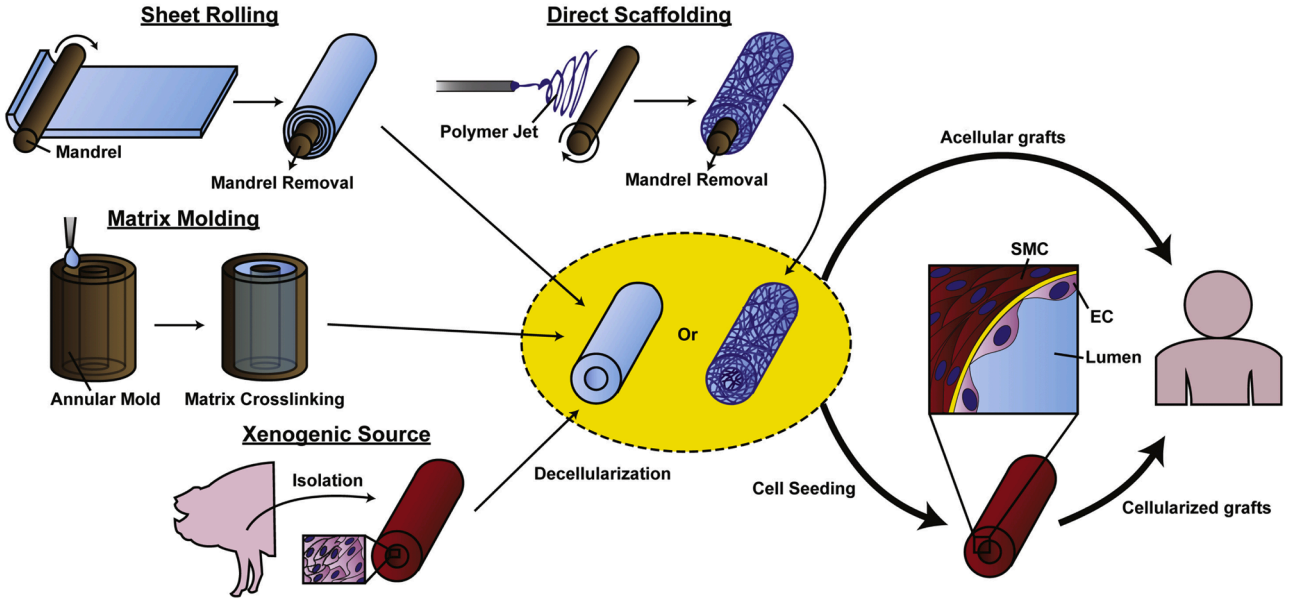

Fig. 3. Fabrication approaches for ETVGs. Tubular structures that resemble small arteries (< 6mm diameter) can be xenogenically sourced and decellularized from animal donors, fabricated with biomaterials through sheet rolling or cell-loaded gel molding, or based on scaffolds made of synthetic or natural materials. Once fabricated, these vessels may be seeded with SMCs, fibroblasts, or ECs, and conditioned *in vitro* through bioreactors before implantation or implanted directly as acellular grafts. Reprinted with permission from Song et al. (2018) Copyright © 2018 Elsevier Inc.

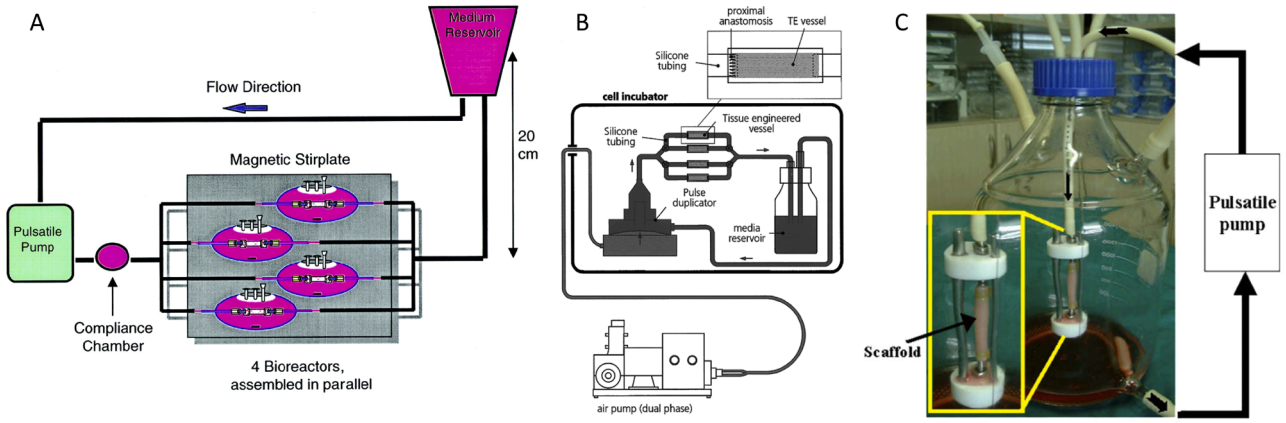

Fig. 4. Three early ETVG bioreactor designs for *in vitro* culture of cell-seeded scaffolds. Adapted with permission from (A) Niklason et al. (1999), (B) Hoerstrup et al. (2001), and (C) Jeong et al. (2005). Copyrights © 1999 The American Association for the Advancement of Science, 2001 and 2004 Elsevier.

cells and without the need for extended culture times in bioreactors (Matsumura et al., 2003); in parallel, self-assembling ETVGs without the use of supporting scaffolds as proposed by L'Heureux just slightly before (L'Heureux et al., 1998, 2006) were not seen as popular and did not see substantial developments by many other research groups as their scaffold-based ETVG counterparts until much later. Hoerstrup et al. (2001) further established the feasibility of small-diameter ETVGs by developing 5 mm diameter non-woven PGA grafts, coated with P4HB, seeded with myofibroblasts for four days and then with endothelial cells, cultured in a bioreactor under pulsatile flow and pressure for up to 28 days (Fig. 4B). The authors obtained viable cellular tissues, with a positive correlation between mechanical training and tissue formation, tissue organization/cell alignment in the flow direction, and sufficient mechanical stiffness (burst pressures reported to be higher than 300 mmHg), but without a confluent endothelial surface remaining present. The benefit of this approach was the ability to subject the luminal surface of the grafts to direct contact with the culture media in the flow loop (instead of sitting on top of silicone tubing as Niklason and co-workers), which in turn may have improved transmural nutrient supply but on the other hand, may have washed-out the seeded endothelium.

Cell seeding, one of the first steps in any scaffold-based ETVG experimental program, has always been a challenge due to the large length-to-diameter aspect ratios of the desired tubular structures, in particular, to deliver cells to the intraluminal surface with the traditional static methods. Diverse and highly innovative methodologies have been developed in parallel with most of the ETVG development efforts over the years. Nasseri et al. (2003) proposed substantial improvements to the rotational seeding technique by introducing a large spindle of 18 cm diameter holding closed culture vessels that circled slowly at 5 rpm and allowed for continuous non-monitored rotation of seeded grafts during long-term culture but necessitated daily media change. Slightly after, in 2004, Williams and Wick (2004) proposed perfusion-seeding through transmural flow through porous nonwoven PGA tubular scaffolds and observed robust smooth muscle cell proliferation.

Over the last 20+ years, scaffold-based approaches introduced many modulating variables and employed many manufacturing techniques, biomaterials, and bioreactor-mediated culture conditions. A large range of hemodynamically-equivalent pulsatile bioreactors were attempted to achieve the overarching goal of attaining ETVGs that resembled a native artery. Iwasaki et al. (2008) have produced 3-layer ETVGs manufactured around a silicone tube serving as a mandrel. Non-woven meshes of PGA were used as templates for seeding SMCs and fibroblasts harvested from bovine sources, which were then wrapped around the silicone tubing with a porous PCL sheet in between forming the bulk of the ETVG wall. After several weeks to complete this process, the silicone tubing was then removed and ECs seeded luminally. These ETVGs were cultured under pulsatile conditions in a pneumatically-driven flow loop that included a left ventricular model with synthetic mitral and aortic valves, with the novelty of delivering realistic pressure and flow waveforms as seen natively to simulate the growth process from fetus to adult. Specifically, throughout a 2-week *in vitro* culture period, mean pressures and flow rates progressively increased from 20 mmHg to 100 mmHg and from 0.2 L/min to 0.6 L/min, respectively. The authors observed a distinctively similar appearance to native vessels, and the ETVG demonstrated good mechanical integrity by recovering its cylindrical shape when deformed and showing appreciable nonlinear behavior (toe-and-heel regions) when tested in the circumferential direction.

Jeong and co-authors in the mid-2000s explored mechano-active *in vitro* culture conditions to modulate the phenotype of VSMCs. Typically, *in vitro* culture of SMCs with conventional techniques are not yet functional as these cells typically revert from a contractile to a synthetic phenotype. The authors have shown that pulsatile strain and shear stress stimulates tissue development, enhancing cell proliferation and collagen production, cells retain their differentiated phenotype expressing α-SMA and myosin heavy chain, and a significant cell alignment in the radial direction similar to native smooth muscle tissues was observed. The authors have attempted blends of polycaprolactone with either polylactide or polyglycolide blocks with an extrusion-particulate leaching technique, resulting in biodegradable scaffolds that were flexible and rubber-like elastic with 90% porosity and an average pore size of 150 ± 50 μm (Jeong et al., 2005). Subsequently, a hybrid scaffold composed of jellyfish collagen and poly(lactide-co-glycolide polymer) obtained by electrospinning and with similar resulting characteristics was employed (Jeong et al., 2007). Cell seeding was achieved with perfusion of cell suspensions through the porous scaffolds, and culture with static or dynamic conditions was conducted for up to 8 weeks *in vitro* (Fig. 4C), with substantial differentiation between culture conditions regarding cellular proliferation and collagen content.

Starting in 2006, Vorp and colleagues have established and refined extensive methods of manufacture and assessment of ETVGs. Currently, this research group is capable of producing and effectively seed-culture-and-implant human-sized vascular grafts with 4.7 mm diameter and up to 10 cm in length (Fig. 5, Cunnane et al., 2020). This is the result of 15+ years of R&D efforts by this group and is now achieved with a complex state-of-the-art seeding apparatus that involves a translational motor responsible for diffusing cell suspensions (of human adipose-derived mesenchymal stem cells) to be seeded intra-luminally along its length, driven transmurally with a differential pressure achieved through a vacuum, while at the same time an additional motor provides continuous rotation as done previously (Soletti et al., 2006; Udelsman et al., 2011). Vorp and colleagues often use porous bi-layered scaffolds made

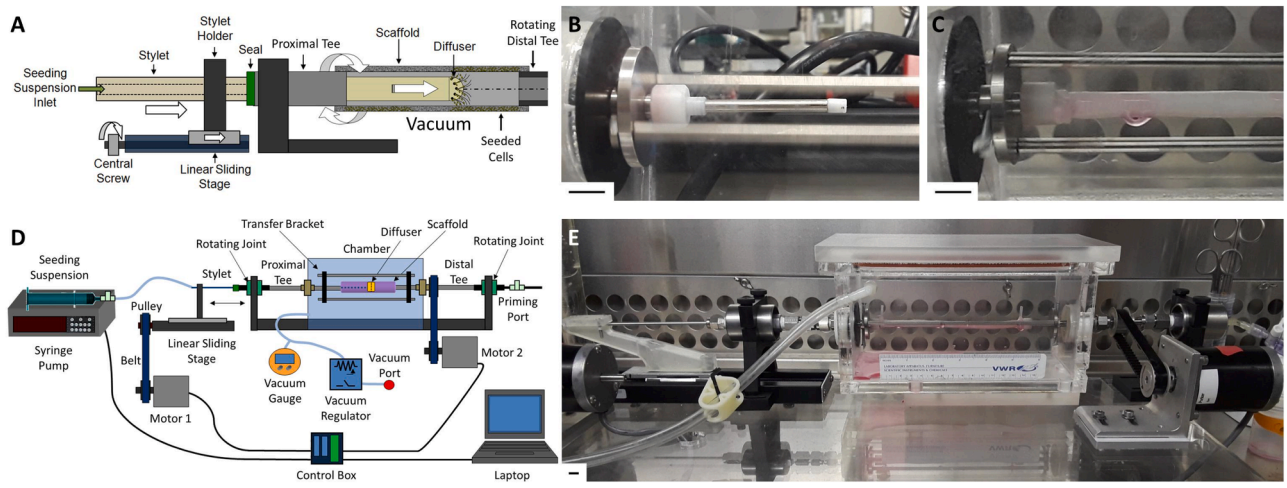

Fig. 5. Overview of the current iteration of Vorp and co-workers seed-and-culture ETVG bioreactor: (A–C) cell seeding is achieved with luminal perfusion with an adventitial vacuum delivered by a sliding diffuser that can cover the entire length of the ETVG; (D, E) schematic and picture of the bioreactor showing the motor to achieve slow rotation of ETVGs during culture. Reprinted with permission from Cunnane et al. (2020). Copyright © 2020 Cunnane, Lorentz, Soletti, Ramaswamy, Chung, Haskett, Luketich, Tzeng, D'Amore, Wagner, Weinbaum and Vorp.

with poly(ester-urethane)urea (PEUU) with a combination of thermally induced phase separation and electrospinning processes (Soletti et al., 2010), which have been extensively characterized in terms of biocompatibility, mechanical properties, microstructure, and ease of manufacture. Over the years, diverse ETVGs were produced with variations of scaffold formulations, different cell sources and seeding methods, and assessment techniques for *in vitro* development and *in vivo* performance. Nieponice et al. employed muscle-derived stem cells seeded through perfusion to create 2 cm long ETVGs with 4 mm diameter and 200 μm thickness and cultured under static conditions inside a spinner flask for 7 days supplemented with ascorbic acid. Seeded cells demonstrated rapid proliferation, retained their stem cell features and produced collagen (Nieponice et al., 2008). Compared with other studies where engineered tissue growth was mainly restricted to the luminal and adventitial surfaces, the authors obtained substantial cell presence transmurally. Mechanical properties were assessed with suture retention strength and uniaxial testing of circumferential rings, and were comparable to values characteristic of the human internal carotid artery (about 60%), although certainly driven mostly by the properties of the scaffolds themselves. To demonstrate *in vivo* deployability, the same approach was subsequently cast into much smaller ETVGs (1 cm long with 1.2 mm internal diameter) suitable for implantation as interposition grafts in the abdominal aorta of rats for 8 weeks for comparison between two scaffold types (with and without the electrospun outer layer) and acellular controls (Nieponice et al., 2010). The authors found that the cellular component significantly improved patency after 8 weeks, and the newly remodeled tissue consisted of aligned collagen fibers, smooth muscle cells, and an endothelial layer. The electrospun outer layer was required for good mechanical performance and to prevent aneurysm formation, but on the other hand, remained mostly non-degraded and its degradation profile required optimization. Subsequently, the same group applied a similar methodology with pericytes and adipose-derived mesenchymal stem cells as an alternative cell source obtaining comparable *in vitro* and *in vivo* results (He et al., 2010; Krawiec et al., 2016; Haskett et al., 2018). Biofunctionalization of the luminal interface with a non-thrombogenic phospholipid to significantly reduce platelet adhesion resulted in off-the-shelf acellular grafts that showed encouraging patency rates up to 6 months *in vivo* and demonstrated the presence of a neo-intima with SMCs and ECs and oriented collagen and elastin deposition (Soletti et al., 2011).

7.2. Cell-laden hydrogel-based ETVGs

Jockenhoevel and co-authors in 2008 attempted the development of a completely autologous small-caliber ETVG by utilizing a bioabsorbable, microporous poly (L/D lactide mesh as a support scaffold system combined with an autologous fibrin cell carrier material with arterial SMCs and fibroblasts and subsequently lined with endothelial cells for an ovine source (Tschoeke et al., 2009). The authors developed a custom-designed manufacturing technique based on casting the fibrin gel in a mold with the polymeric mesh. The resulting composites of polymeric mesh with cell-laden fibrin gels inside an outer silicone tube (which served as the mold) were cultured in a bioreactor circuit for dynamic mechanical conditioning holding three samples over a 21-day *in vitro* culture period. Flow and pressure progressively increased to 200 mL/min and 120 mmHg during the culture period. Burst pressure of approximately 400 mmHg was observed after 7 days of *in vitro* culture without further increases, while collagen content and viable cell number increased up to 21 days.

Feijen and co-workers have employed tubular scaffolds (3 mm inner diameter and 4cm in length) composed of cross-linked insoluble collagen type I and elastin seeded with SMCs through filtration seeding and cultured under pulsatile conditions similar to the environment found in the human carotid artery. This culture system allowed for four grafts to be co-cultured under the same conditions, each one inside its bioreactor, but all sharing the same culture media. Pressure pulses were gradually increased from 30 beats per minute up to 120 beats per minute, with magnitudes of 61 to 124 mm Hg diastole-to-systole. The authors observed a substantial increase in VSMC proliferation and mRNA expression attributed to the dynamic environment. Additionally, different crosslinking agents were compared not only in terms of their effects on the mechanical properties of the original scaffolds (Buttafoco et al., 2006) but also on the biological response they would trigger during the *in vitro* culture of VSMCs (Engbers-Buijtenhuijs et al., 2006). Several years after, Feijen and co-workers investigated poly(trimethylene carbonate) (PTMC) porous tubular scaffolds with similar dimensions (3mm diameter, but with 1mm wall thickness and up to 8 cm long) obtained with salt-leaching and coated with an outer layer with lower porosity to increase VSMC seeding efficiency by decreasing pore size from 110 μm to 28 μm and porosity from 85% to 65%. VSMCs were delivered from the lumen through the wall through perfusion-seeding (Song et al., 2010). These ETVGs were cultured for up to 14 days in a Bose-Electroforce pulsatile flow bioreactor operating with three independent culture channels in parallel with the flow pulse delivered by a

linear displacement pump programmed to mimic the flow environment of the human carotid artery with intraluminal pressures ranging from 70 to 130 mmHg at 69 beats per minute (Song et al., 2011). Subsequently, this group attempted a departure from using off-the-shelf pulsatile bioreactors towards custom-design bioreactors (Song et al., 2011) to achieve similar effects. This new system was based on the original design of Webb et al. (Webb et al., 2007), with the additional advantage of being able to measure compliance non-destructively during culture with optical measurements of ETVG diameter with a LED micrometer. The authors found, similarly to their previous studies, that cell number was significantly higher with dynamic conditioning, indicating that improved transport of nutrients and waste products and/or cyclical mechanical strain (the outer diameter of ETVGs with pulsatile flow increased approximately 7%) stimulated SMC proliferation directly, increased collagen I expression at the mRNA level, and yielded a substantial increase in mechanical stiffness in the axial direction when compared with statically cultured ETVGs (Song et al., 2011).

Hahn and colleagues have produced ETVGs based on diacrylate-derivatized polyethylene glycol (PEG) hydrogels. PEG hydrogels have diverse characteristics that make them highly desirable for vessel replacement biomaterials, such as biocompatibility and non-thrombogenicity. The gel can be photopolymerized in the presence of cells, from which tubular constructs with homogeneously seeded cells can be formed in appropriately shaped molds (Hahn et al., 2007), and provide the added benefit of being easily modifiable with bioactive moieties. Additionally, the resulting PEG-based biomaterials are highly elastic and their mechanical properties tunable. Hahn and co-workers have developed PEG-based ETVGs *in vitro* with custom-designed bioreactors to investigate the effects of pulsatile flow (with diverse pulse waveforms, magnitude, and frequency) and the circumferential strain it induces on tubular constructs. As cell sources, the authors have investigated the employment of mouse embryonic progenitor cells included in the hydrogel before polymerization, and the inclusion of a "cemented" endothelial cell layer with bovine aortic ECs lining its luminal surfaces (Jimenez-Vergara et al., 2010). In addition, the authors have explored diverse options to be added to the PEG formulation, such as the inclusion of fibrin or a collagen-mimetic protein derived from group A Streptococcus (an attractive alternative to solid-phases synthesis of ECM peptides, McMahon et al., 2011; Browning et al., 2012). Subsequently, Hahn and co-workers have explored options for improving the mechanical stiffness of the PEG-based ETVGs with the addition of an electrospun component. Polymers employed for the electrospun outer layer were an alternating poly (e-caprolactone) soft segment and a urethane- and urea- containing hard segment (McMahon et al., 2011), and segmented polyurethanes (Browning et al., 2012; Post et al., 2019). ETVGs are generally cultured in *in vitro* systems in biomimetic environments where dynamic culture conditions can be achieved with pulsatile flow. More recently, Hahn and co-workers have investigated the effects of host vessel-ETVG compliance mismatch with the inclusion of two segments of excised porcine carotids on which the graft ends are anastomosed *in vitro* with this novel bioreactor design (Post et al., 2019).

The Tranquillo research group has made significant contributions to diverse areas of cardiovascular tissue engineering and has achieved substantial progress in the ETVG arena based on fibrin gel technology as an alternative biopolymer (compared with collagen gels) for tubular scaffolds that do not suppress ECM synthesis by VSMCs and fibroblasts and promote an optimal environment for de novo tissue formation and strength (Neidert et al., 2002; Grassl et al., 2003). These fibrin-based SMC-remodeled media-equivalents were capable of being successfully endothelialized with full surface coverage, expression of von Willebrand factor, and retention of EC when exposed to relevant physiological shear stresses (Isenberg et al., 2006). One of the disadvantages of not employing a synthetic scaffold for mechanical support is the lack of mechanical properties, which may render fibrin- or collagen-based ETVGs not suitable for implantation - in 2010, Huynh and Tranquillo (2010) demonstrated the feasibility of layering fibrin-based mature engineered tissue sheets promoting tissue fusion and obtained a substantial increase in achieved mechanical stiffness, ETVG integrity, and reported burst strengths up to 600 mmHg. Subsequently, fibrin-based fibroblast-seeded ETVGs with diameters of 2 and 4 mm were cultured for two weeks under static conditions. The ETVGs then transitioned into a custom-designed bioreactor (allowing for six grafts in parallel, Fig. 6 left panel) for dynamic culture under pulsatile flow conditions for up to 9 weeks with 0.5 Hz frequency and peak circumferential strains of 5 to 10% (Syedain et al., 2011). The increased burst strength was substantial and deemed sufficient for subsequent *in vivo* implantation, reaching up to 1500 mmHg, and correlated to increases in collagen concentration and circumferential alignment. Tranquillo and co-workers have carried these ETVGs into the *in vivo* setting in the ovine and the primate models (Syedain et al., 2017, 2014), but with the particular characteristic of including a decellularization step to implant a non-living but biological graft following a similar approach employed by Dahl et al. (2011). SDS and TritonX-100 detergents were able to decrease DNA content up to 93% with negligible loss of mechanical properties before implantation. After 24 weeks, the acellular ETVGs did not elicit a chronic immune response and remodeled appropriately without aneurysm formation, with only rare CD45 positive cells being present and consistent with the hypothesis that favorable remodeling ensued after the resolution of initial inflammation due to the reduced immunogenicity of these grafts. The ETVG was indeed able to recruit host cells, positively expressing α-SMA and calponin indicative of SMC maturation, stained negative for mineralization and positive for elastin with strong circumferential alignment. In a separate study, the incorporation of an autologous endothelium was demonstrated and compared with bare ETVGs (Meier et al., 2014). Although the employment of autologous endothelialization delays clinical availability and complicates commercialization and translation of ETVGs, it adds potential benefits for reducing acute thrombogenicity and promoting full endothelialization, for inducing a precursor of a basement membrane facilitating cross-talk between ECs, and medial VSMCs/fibroblasts, and for providing additional positive cues for favorable remodeling and reduction of intimal hyperplasia. Successes with the original ovine model were translated to preclinical studies for a vascular graft capable of growth suitable for hemodialysis access as AVF grafts tested in baboons for up to 6 months (Fig. 6, middle panel, Syedain et al., 2017), and for congenital heart disease corrective surgery implanted for up to 42 weeks in growing lambs (Fig. 6, right panel, Syedain et al., 2016).

7.3. Self-assembled ETVGs

The earliest report of a self-assembled ETVG dates back to 1993 by L'Heureux et al. (1993). A vessel "equivalent" to a native artery was constructed *in vitro* with three layers of medial VSMCs and luminal endothelium (from human umbilical vein) and adventitial fibroblasts (from human skin). Initially, a media-like structure was first obtained from the contraction of a tubular collagen gel seeded with VSMCs, followed by adding adventitia-like tissue embedding fibroblasts, and lastly, luminally seeded with endothelium. These early ETVGs were initially developed as an *in vitro* model for pharmacological studies, suitable to investigate phenomena such as VSMC migration, proliferation, and contractility (L'Heureux et al., 2001), or the cross-talk between ECs and VSMCs (Stoclet et al., 1996). The novelty and significance of L'Heureux and co-workers approach at the time was indeed the lack of any synthetic or exogenous biomaterials needed for the construction of the self-assembled structure; however, its biggest drawback was certainly the maturation time required (up to 3 months, L'Heureux et al., 1998). Over the next few years, the technique was simplified and refined into just obtaining sheets of cells (VSMCs and fibroblasts) and their deposited ECM during 30 to 45 days of standard culture. The authors investigated and found the optimal strength to culture time ratio – after which these sheets could be manually peeled off and used to manufacture ETVGs. The ETVG started with an acellular inner membrane made from a

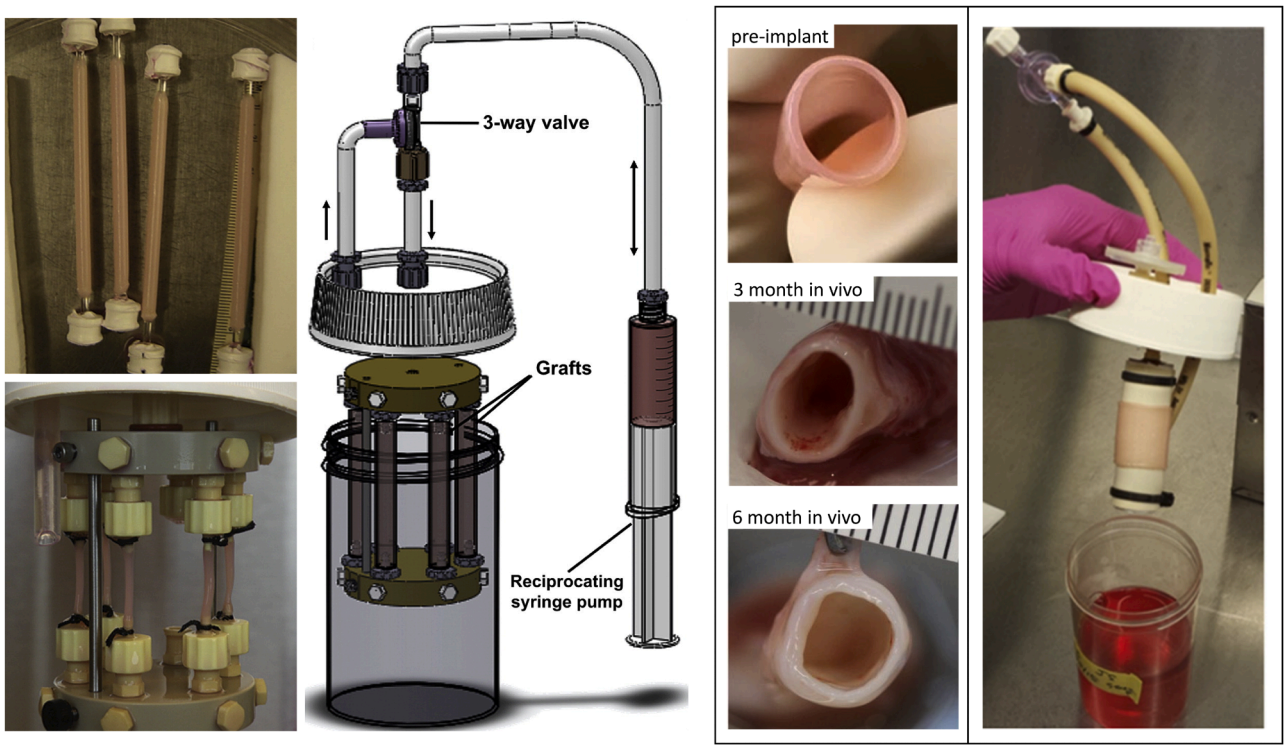

Fig. 6. (Left panel) Tranquillo and co-workers pulsed flow-stretch bioreactor in 2011. ETVGs made with fibroblast-seeded fibrin gels are cultured for 2 weeks statically, then transferred into a bioreactor that delivers culture media axially and transmurally; **(middle panel)** end-on view of 6-mm diameter decellularized ETVGs before implantation in the baboon model of hemodialysis assess, and after 3 and 6 months in vivo; **(right panel)** subsequent version of Tranquillo and co-workers in 2016 for culturing large diameter (up to 2 cm) ETVGs. Reprinted with permission from Syedain et al. (2011, 2017), and Schmidt and Tranquillo (2016). Copyrights © 2010 Elsevier, 2017 The American Association for the Advancement of Science, 2015 Biomedical Engineering Society.

fibroblast sheet wrapped around a perforated Teflon tubular mandrel with a 3-mm diameter. Then, a VSMC sheet was rolled around to create a media layer and the construct is placed on a bioreactor to provide a luminal flow of culture media. After 1 week of maturation, the fibroblast sheet was then wrapped around the construct followed by 8 more weeks of maturation, after which the supporting mandrel could be removed. The last step, luminal endothelialization (when employed) used standard methods and achieved full coverage of the acellular inner membrane (the technique was continued by Auger and Germain, original collaborators of L'Heureux at Laval University, with the method shown in Fig. 7). The ETVGs revealed well-defined layers: intima, media, and adventitia. The adventitia exhibited very dense collagenous layers as well as abundant fibroblasts. In the media, VSMC appeared as elongated cells with circumferential or longitudinal orientations, staining positively for α-SMA and desmin and capable of developing active tension and did not penetrate the inner membrane separating lumen and media. Adventitial fibroblasts expressed vimentin and synthesized high amounts of elastin assembled in small fibers, which were organized in large circular arrays. ECM was mostly composed of collagen types I, III, and IV, as well as laminin and fibronectin. Mechanical integrity stiffness was carried mostly by the optimized adventitia, reaching burst pressures of 2200 mmHg after 7 weeks of culture and plateaued at these valuers after. Ultrastructural analysis of the adventitial sheets revealed long collagen fibrils, featuring the characteristic cross-striation D-periodicity, organized in closely packed bundles of parallel fibrils orientated perpendicular to one another, with a network of elastin-associated microfibers parallel to the collagen fibrils, characteristic of mature dense collagenous tissues.

With such promising results *in vitro*, the next logical step was to try them *in vivo* – ETVGs were employed as a femoral bypass in dogs, non-endothelialized to avoid the acute rejection of xenogeneic ECs. After 7 days, patency was 50% (3 out of 6 failed due to acute thrombosis), but patent grafts retained tissue architecture and did not show any sign of degradation, tearing, or dilatation. Several years after, L'Heureux and co-workers, now at Cytograft Tissue Engineering Inc, reported successful long-term implantations of ETVGs made with the acellular internal membrane and only the fibroblast sheets (eliminating the need for SMC layers) as abdominal aortic interpositional grafts in rats for up 225 days and in primates up to 8 weeks, or as end-to-end iliac interpositional grafts in primates up to 6 weeks (L'Heureux et al., 2006). The mechanical properties of these ETVGs were outstanding and most importantly, demonstrated that no scaffold is truly necessary to confer mechanical integrity to ETVGs, at least in the long term (Konig et al., 2009). Shortly after, L'Heureux and co-workers reported the first human trial of the employed of their sheet-based ETVG made from autologous cells with 9 patients with failing hemodialysis AV shunts (McAllister et al., 2009; L'Heureux et al., 2007). Three grafts failed during the initial 3 months because of graft dilatation, aneurysm, or thrombosis; one patient had non-related death, while five remaining grafts were able to continue dialysis for more than 6 months. To target patient populations or clinical applications that cannot tolerate long production times associated with the fully autologous ETVG technology, L'Heureux et al. shifted efforts to using allogeneic tissues to create "off-the-shelf" ETVGs. In 2011, L'Heureux and co-workers reported the employment of a decellularized version of their ETVG technology that allows long-term storage – five days before implant and an AV shunt, the devitalized biomaterial is rehydrated, and only autologous ECs are seeded luminally to provide an anti-thrombogenic lining (Wystrychowski et al., 2011). These allogeneic AVGs were remarkably strong: they possessed > 5000 mmHg burst pressure and >250 g suture retention strength pre-implantation and were tested for functionality with puncture for hemodialysis after 2-3 months of AVF maturation. The grafts could withstand the harsh environment of the AV shunt without signs of an aneurysm for up to 11 months; however, cells remained absent,

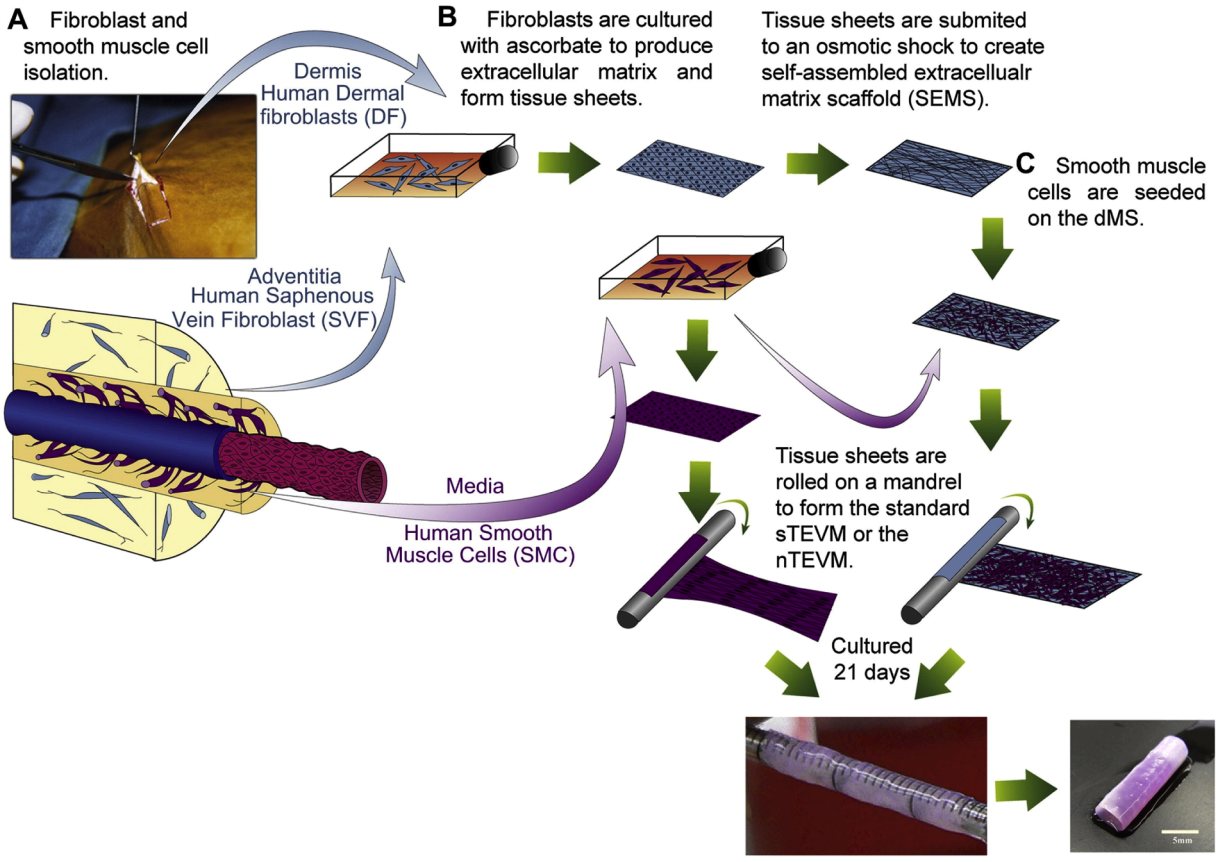

Fig. 7. Schematic view of the processes required for the production of cell-based ETVGs. Human fibroblasts are isolated from skin or SV biopsies, and SMCs are isolated from the media of the umbilical artery and cultured, resulting in tissue sheets that are then decellularized and rolled. Reprinted with permission from Bourget et al. (2012). Copyright © 2012 Elsevier.

adversely affecting graft performance and hampering the repair process necessary at puncture sites. No further trials of Cytograft ETVGs were conducted and it is unclear if further trials will be undertaken (Chang and Niklason, 2017). L'Heureux left Cytograft in 2015 and over the recent years, L'Heureux and co-workers have shifted focus towards the development of cell-assembled ECM either in the form of sheets or yarns that can be assembled into "human" textiles suitable for diverse tissue engineering applications (Magnan et al., 2018, 2020).

Over the years, not many others have employed self-assembled cell-sheet techniques for the creation of ETVGs, potentially because of the complexity of the method. Some examples of self-assembled ETVGs were proposed by Auger and Germain (Fig. 7, Bourget et al., 2012; Gauvin et al., 2010; Vallières et al., 2015), Chaikof and co-workers (Kumar et al., 2013), Othman et al. (2015), Feng Zhao and co-workers (Xing et al. 2017, He et al. 2022), and Jie Zhao et al. (2012, 2013). On the other hand, several other techniques can be considered under the umbrella of self-assembled ETGVs. Microtissue aggregation avoids the peeling processes and uses templates for culture, which could be similar to molding/casting with cell-laden gels (Kelm et al., 2010). Lastly, 3D-bioprinting of ETVGs has been reported as a potential manufacturing process but still has significant limitations (Norotte et al., 2009; Melchiorri et al., 2016; Fukunishi et al., 2017), and certainly will become a very important manufacturing process as 3D-bioprinting matures as a technology and printing resolution increases shortly (Duan, 2016).

8. ETVGs on the cusp of clinical translation

Besides efforts by Tranquillo and co-workers with preclinical studies in large animals (Syedain et al., 2017, 2016), and L'Heureux and co-workers with trials with Cytograft (McAllister et al., 2009; L'Heureux et al., 2006; L'Heureux et al., 2007), two ETVGs are currently very close to finally achieving the ultimate goal of potential clinical translation.

8.1. Humacyte human acellular grafts for hemodialysis access and PAD grafting

Niklason and co-workers have historically been pioneers of ETVG technology since its early days. Humacyte, founded by Niklason in 2004, has been a remarkable success story of biotechnology entrepreneurship and has incorporated the immense body of research into its goals of reaching clinical translation of ETVGs. After several years with financing status backed by investors, the company is now publicly held in the NASDAQ index and is developing off-the-shelf bioengineered tissues to treat unmet clinical needs in various applications. Humacyte's most advanced product candidate, Human Acellular Vessels (HAV), is in late-stage clinical trials targeting trauma repair, AVF access, and peripheral artery disease. Humacyte HAVs have the prospect of FDA approval and large-scale manufacture and commercialization plans within the next two years (Fig. 8, bottom panel). Niklason et al. (1999) first reported ETVG development with a scaffold-based approach with a dynamic *in vitro* culture environment achieved with fluid flow operating at 165 bpm and a pressure pulse ranging from 30 mmHg to 270 mmHg resulting in approximately 5% diameter changes (Fig. 4, . ETVGs were cultured from bovine aortic SMV seeded in a 5-mm internal diameter PGA mesh scaffold inside a Dacron sleeve for up to 8 weeks and allowed for subsequent luminal seeding with endothelium. Niklason et al. observed a high degree of SMC organization in a highly lamellar structure, with cells separated by alternating layers of collagen fibrils. Mechanically conditioning throughout *in vitro* incubation resulted in a substantial improvement of mechanical strength, reaching comparable values to

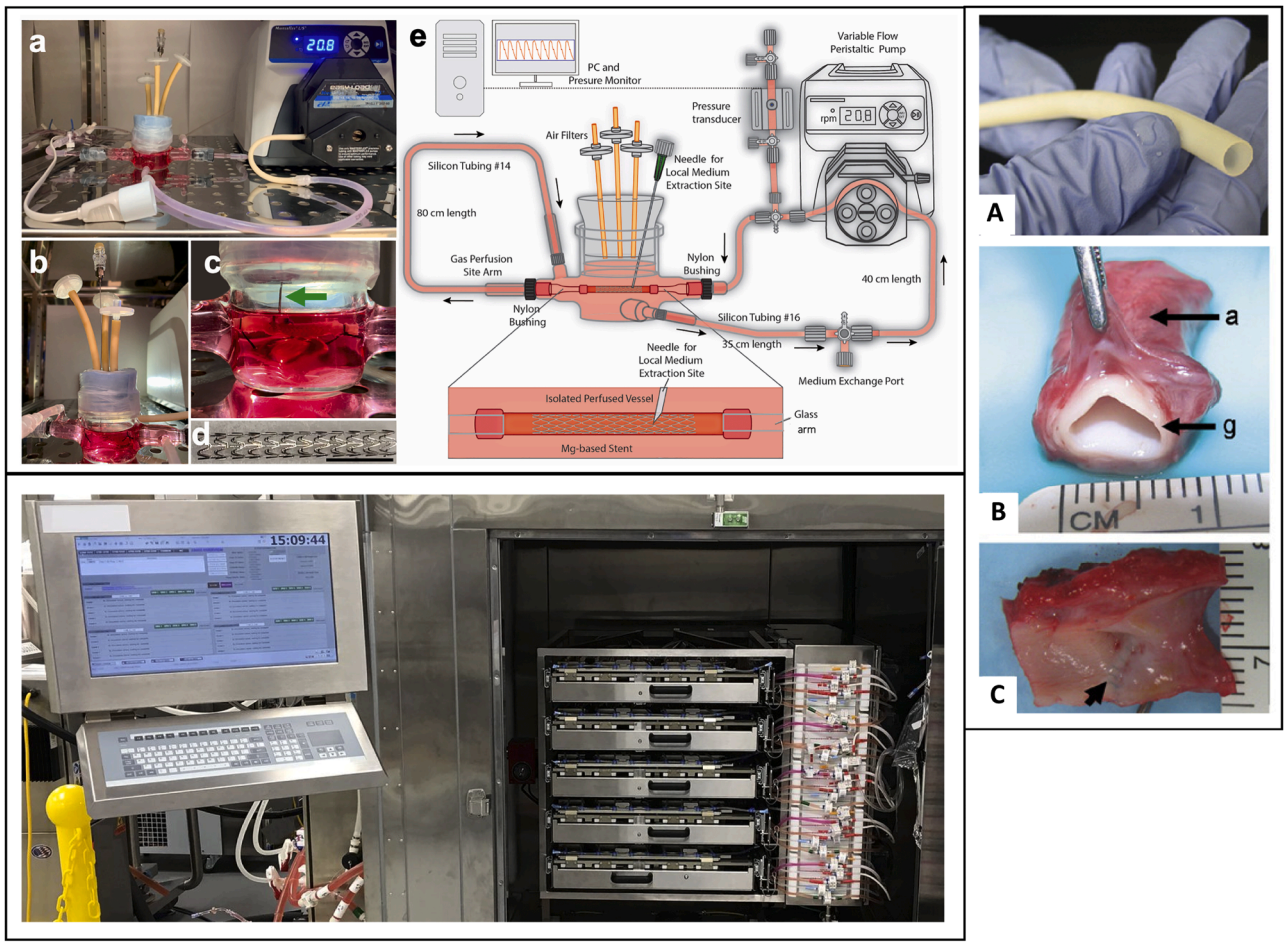

Fig. 8. (Top panel) most recent version of Niklason et al. ETVG bioreactor for research purposes, here shown as a vessel culture bioreactor setup for the investigation of bioerodible magnesium stent, reported by Wang et al. (2021) This bioreactor design is based on previous work of Huang et al. (Dahl et al., 2007a, 2007b, 2008), which allowed for axial stretch besides pulsatile stretching; (right panel) 6-mm diameter decellularized ETVG before implant (A), and explanted from the baboon model for hemodialysis access after 6 months, showing de novo adventitia and original graft (B and C) reported by Dahl et al. (2011); (bottom panel) ETVG state-of-the-art technology at Humacyte with automated and self-contained systems for culture and decellularization of ETVGs. Adapted with permission from Dahl et al. (2011), Wang et al. (2021), Niklason and Lawson (2020). Copyrights © 2011, 2020 The American Association for the Advancement of Science, 2021 Elsevier.

native arteries (although much lower than gold-standard, the saphenous vein burst pressure). The degrading scaffold did not contribute to the mechanical stiffness, but ETVG contractility measured through exposure to pharmacological stimuli was only a fraction of that of native vessels, possibly due to the lack of elastin (Niklason et al., 2001). A fraction of highly mitotic SMCs was observed in the vicinity of PGA fragments, which could become problematic for recruiting leukocytes expressing growth factors characteristic of an *in vivo* inflammatory FBR. Thus, the logical next step was to attempt to improve/optimize the scaffold to minimize or mitigate risks of hyperplasia and graft occlusion after implantation (Prabhakar et al., 2003). At the time (and indeed still today), the duration of the *in vitro* culture period was considered a substantial drawback of any ETVG technology, with some research groups reporting culture times requiring up to 4 months to achieve blood vessels with sufficient mechanical integrity. To mitigate these issues, Dahl et al. proposed the employment of decellularized native arteries as a biological scaffold for either autologous cell seeding for an initial period of *in vitro* remodeling, or even implanted as an acellular graft and relying on host SMC migration (Dahl et al., 2003). This approach had been attempted and developed before the ETVG-paradigm existed as an alternative to synthetic vascular grafts, e.g. Badylak and co-workers research on employing small intestinal submucosa dating back to 1989 (Lantz et al., 1992; Badylak et al., 1989). For several years, Dahl et al. extensively characterized the PGA-based ETVGs obtained from VSMCs under dynamic incubation, certainly the necessary steps to fully harness the manufacturing process with the investigation of different modulators of the process (Solan et al., 2003). Establishment of the feasibility of expanding cells from elderly human donors was established (McKee et al., 2003; Poh et al., 2005), ETVG composition and mechanical properties were carefully measured and reported (Dahl et al., 2007a), de novo collagen ultrastructure was extensively characterized (Dahl et al., 2007b), and a growth and remodeling framework was proposed for de novo engineered tissue formation based on these results (Dahl et al., 2008). Building upon these experiences, Niklason and co-workers initiated their current approach to develop acellular ETVGs obtained from decellularized engineered tissues formed on fast-degrading PGA-scaffolds.Reports of successful animal implantation by Niklason and co-workers happened in 2011 in another landmark contribution to the ETVG field – Dahl et al. (2011) reported the strength and stability of decellularized ETVGs made with VSMCs (obtained from cadaveric donors or from canine vasculature) on rapidly degradable PGA tubular scaffolds cultured in bioreactors subjected to cyclic luminal pressurization for 7 to 10 weeks of culture. After *in vitro* culture, resulting grafts were decellularized with detergents, and stored at 4 °C up to 12 months without any reduction in mechanical properties. Human-derived ETVGs were implanted in baboons (to provide phylogenetic similarity to humans, which allowed for implantation without immunosuppression) as an AVF model, and canine-derived ETVGs were

endothelialized with autologous cells before implantation and deployed end to side as peripheral carotid artery bypass in the canine model. Results after one year were outstanding, with patency of 88% in the baboon model and 83% in the canine model (7/8 and 5/6, respectively). No onstrictive fibrotic tissue surrounding the grafts and any immunogenic response (i.e. no calcification, T-cell, or foreign body giant cells proliferation) was observed; but instead, grafts became compositionally more similar to native arteries (Fig. 8 right panel). Elastin was found in anastomotic regions that recruited the most cells (either VSMCs or myofibroblasts), which expressed α-SMA. Actin-positive cells appeared to infiltrate transmurally from the adventitial-like tissue layer. Von Willebrand expression was found in the luminal layer even in the baboon grafts (that were not endothelialized before implantation), indicating the presence of a functional endothelium that may have migrated from anastomosed vascular tissue, transmurally, or originated from circulating progenitor cells. Mechanical properties of the ETVGs explanted from baboons showed no difference from pre-implantation values. Following these successful results, several clinical trials in humans have been conducted and are currently underway to assess the performance of this technology in the clinical setting, specifically as an AVF graft (Lawson et al., 2016; Kirkton et al., 2019; Jakimowicz et al., 2022), and as a peripheral artery bypass graft (Gutowski et al., 2020).

In parallel to the pursuit of clinical translation, Niklason and co-workers have been pushing other boundaries in ETVG technology. Huang et al. (Huang and Niklason, 2011; Huang et al., 2015; Huang et al., 2016) have developed a bioreactor system that can impose axial stretch to ETVGs besides the circumferential stretch due to luminal pressurization. With this system, the authors demonstrated that biaxial mechanical stimuli could indeed be favorable to induce more and better de novo tissue formation. Wang et al. (2021) have recently proposed the subsequent iteration of this bioreactor system into a flow-tunable vascular bioreactor able to maintain normal and hyperplastic porcine coronaries or human atherosclerotic coronary arteries ex vivo for up to 7 days to evaluate bioerodible magnesium stent degradation (shown in Fig. 8 top panel). Additionally, Niklason and Qyang have investigated the employment of immunologically modified human-induced pluripotent stem cells as an alternative source for deriving vascular cells in large quantities for ETVG generation (Gui et al., 2016; Luo et al., 2020, 2022).

8.2. *Vascular conduits for pediatric implantation in the Fontan operation*

Over the last two decades, Breuer and co-workers have established an immense body of work on ETVGs, focusing specifically on *in vivo* function. In the late 1990s and early 2000s, Shinoka and Hibino successfully developed large diameter ETVGs based on PGA-PLA-PCL scaffolds seeded with a mixture of autologous cells in several animal models, specifically sheep and dogs (Watanabe et al., 2001; Shinoka et al., 1998). Promising results were quickly transitioned into human intervention with ETVGs made with autologous bone marrow-derived mononuclear cells (BM-MNCs) seeded onto a biodegradable tubular scaffold fabricated from a PGA fiber–based mesh coated with a 50:50 copolymer of polycaprolactone and PLA (Fig. 9). The first implantation of this ETVG was reported in 2001 in a 4-year-old girl with single ventricle and pulmonary atresia reconstructing the occluded pulmonary artery (Shin'oka et al., 2001); and soon after, reports of the same procedure done on 22 more pediatric patients with single ventricle physiology followed in a pilot study in Japan (Matsumura et al., 2003). Interestingly, these ETVGs were truly a success at the first attempt, and this breakthrough investigation and its outstanding results certainly were responsible for creating the entire ETVG paradigm of today. Post-operative examinations revealed good performance without dilatation or rupture of grafts or complications associated with the ETVGs but were recognized by the authors as limited due to the lack of serial evaluation allowed in a human study (only catheterization, ultrasound MRI and CT scans available, and patient follow up happened one month and then only two years after implantation, Shinoka et al., 2005). Regardless, late results after a mean follow-up of 5.8 years with ultrasonography, angiography, and CT and MR imaging, were outstanding

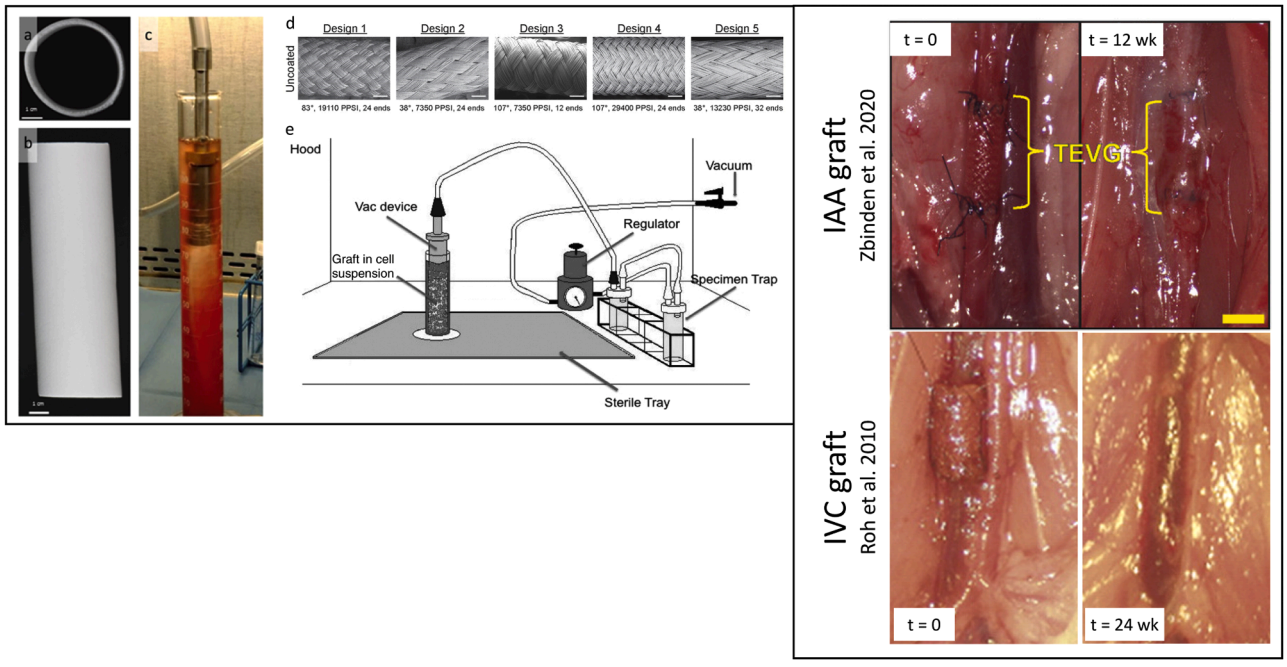

Fig. 9. (Left panel) Shinoka, Breuer and co-workers ETVG scaffold made of knitted PGA fibers coated with 50:50 copolymer solution of PCL and PLA before implantation, 12–20 mm diameter, 1mm wall thickness, 13 cm length (a,b) suitable for vena cava implantation in an ovine model, (c,e) seeded with bone-marrow mononuclear cells through vacuum seeding for transmural delivery. Breuer and co-workers have investigated many manufacturing parameters, e.g. brading designs of scaffold fibers as illustrated in (d); **(right panel)** Breuer and co-workers research modus operandi and key focus has been the deployment of ETVGs in diverse animal models, particularly mice models, where skilled microsurgeons deploy ETVGs as aortic or IVC interpositional grafts and extensively characterize them after explant. Adapted with permission from Pepper et al. (2017), Zbinden et al. (2020), Roh et al. (2010), and Dean et al. (2012).

and revealed no evidence of aneurysm formation, ETVG rupture, infection, or calcification. However, one patient demonstrated mural thrombosis resolved with warfarin, and 7 of 25 patients had graft stenosis (considered the primary mode of graft failure), but all underwent successful percutaneous angioplasty (Hibino et al., 2010). Given the promising results of the Japan trial and the further refinement of the technology with large animal models (e.g., the landmark study by Breuer et al. in 2008 showing growing potential in juvenile lambs (Breuer et al., 2008)), Breuer and coworkers initiated a clinical trial in the US to evaluate these ETVGs under the regulation of the US Food and Drug Administration (FDA); unfortunately, this study was placed on hold only after 4 pediatric implantations due to high incident of early graft stenosis.

Since then, Breuer and co-workers have developed a comprehensive research program that employs combinations of *in vitro, in vivo* (either animal models or human trials), and in silico methods to develop better ETVGs and improve the understanding of their bio-chemo-mechanical processes (Patterson et al., 2012). In 2020 Drews et al. reported that computational modeling of the growth & remodeling processes of *in vivo* function of ETVGs during their inflammatory-mediated integration (based on frameworks of Humphrey and co-workers (Miller et al., 2014, 2015; Szafron et al., 2018, 2019; Khosravi et al., 2020)) "predicted" and suggested that the acute stenosis observed in the US-FDA would have resolved without intervention (and it was not observed in the Japanese trial because of the lack of consistent, early medical imaging) (Drews et al., 2020). More recently, Schwartz et al. conducted patient-specific computational fluid dynamics simulations with data acquired on the 4 patients of the US-FDA trial and demonstrated that graft remodeling occurred in two distinct phases: early and rapid changes in graft geometry occur acutely after implantation and then are followed by a second phase of sustained growth and decreased graft stiffness (Schwarz et al., 2021). Additionally, the authors demonstrated that changes in ETVG geometry, thickness, and stiffness affected patient-specific hemodynamics but remained within normal ranges despite the clinically observed graft narrowing. Although helpful insight can and has been obtained from in silico models, it must be noted that these modeling approaches are still rather simplistic and are potentially inadequate to fully describe/capture the full gamut of the *in vivo* growth and remodeling process of the inflammatory foreign body reaction that ensues after implantation. Issues with typical assumptions of homogeneity, isotropy, their multi-component microstructure that evolves, the interplay between reactive constituents, and the *in vivo* loading state and boundary conditions, render the current state-of-the-art of modeling efforts far from truly realistic (when compared with other much more well-understood chemo-mechanical processes, Rajagopal and Rajagopal, 2020).

In parallel to human trials, Breuer and co-workers have established immense supportive experimental evidence of the *in vivo* performance of their ETVG design with animal models (Fig. 9). Although the design and methods of manufacture of the ETVGs have not changed substantially in concept since the first attempts made by Shinoka, many efforts investigating a wide range of aspects associated with ETVGs have been conducted. Earlier efforts were targeted at obtaining a deeper understanding of the processes of ETVG manufacture and the *in vivo* remodeling post-implantation, specifically optimization of seeding density (Roh et al., 2007a, 2007b), methods to achieve operator independence on seeding (Udelsman et al., 2011; Kurobe et al., 2015), diverse imaging techniques for ETVG assessment (e.g., phase contrast MRI, Stacy et al., 2018, intravascular ultrasound,Pepper et al., 2017), understanding the fate of seeded-cell and the process of host-cell recruitment (Hibino et al., 2011a; Harrington et al., 2011b), and the inflammatory FBR triggered by ETVG implantation (Roh et al., 2010; Hibino et al., 2011). More recently, pinpointed investigations specifically aimed at refining/improving their modus operandi have been attempted and published (most often in small animals, mice or rats, or large animal models, typically sheep). These investigations focus on several features of their method on diverse measurable aspects of the *in vivo* response to vascular grafting (Fukunishi et al., 2020). Examples of such are the choice of different scaffold materials, e.g., small diameter sponge-type scaffolds suitable for arterial bypasses made of PLA-PCL reinforced with PLLA (Kurobe et al., 2015; Sugiura et al., 2016) or scaffolds with a compliant inner core of poly(glycerol sebacate) with a stiffer sheath of PCL (Wu et al., 2021), or the effects of different braiding patterns (Zbinden et al., 2020), pore sizes (Matsuzaki et al., 2020), degradation rates/profiles (Fukunishi et al., 2020), or even bulk dimensions of the ETVGs themselves (Best et al., 2018). Two typical problems persist and remain to be solved – acute occlusion due to thrombosis when diameters are small and the grafts are deployed as an arterial bypass, or mid-to-late term stenosis when serving as cavopulmonary graft possibly due to insufficient mechanical support of the degrading scaffold and/or chronic fibrotic encapsulation triggered by the inflammatory FBR. Many attempts and efforts have been tried to address these issues, e.g., heparin conjugation (Matsuzaki et al., 2021) or administration of Interleukin-10 (Mirhaidari et al., 2020) to prevent thrombosis; and treatment with angiotensin II type 1 receptor inhibitor (losartan, de Dios Ruiz-Rosado et al., 2018), TGFβR1 inhibitors (Duncan et al., 2015; Lee et al., 2016), or cilostazol (Tara et al., 2015), to mitigate adverse remodeling leading to stenosis.

9. Concluding remarks

Clinical results of ETVG performance as AVFs and in cavopulmonary grafting in pediatric reconstructive surgery are encouraging, demonstrate that engineered tissue blood vessels are indeed very close to reaching clinical reality, and we may be entering a new era of therapy for vascular disease (Chang and Niklason, 2017). This has been achieved through the immense empirical effort of many research groups, which has now progressed from research based in academic laboratories into product R&D in the biotechnology industry. However, progress toward widespread employment, production, and commercialization faces translational challenges under existing regulatory frameworks, particularly concerning the employment of autologous cells. A regulatory and ethical debate is necessary to prevent highly conservative positions that may hinder future progress and to not overburden research and development with overzealous concerns of a zero-risk stance requiring too much data to establish the quality, safety, and efficacy of cell-based ETVGs (McAllister et al., 2012).

Regardless, after more than 30 years after the inception of ETVGs and even with the outstanding potential of relieving such critical supply limitations, their performance has not been sufficient to push them to direct clinical applicability. Advances in the field have been slow, incremental, and limited partly due to the lack of systematic understanding of the diverse biological and biophysical factors affecting engineered tissue formation during *in vitro* incubation and remodeling after *in vivo* implantation; in particular, on how cells establish, maintain, remodel or repair the ECM that dictates the overall structural integrity of the graft and the micro-environment they respond to. As of today and as similarly as 20 years ago, ETVGs remain promising, but not yet successful, alternative conduits for vascular grafting. Most of the related advances have been realized by trial-and-error, however, mainly from the painstaking empirical evaluation of a multitude of different construct biomaterials, cell sources, culture conditions, and so forth (Miller et al., 2014).

Three significant challenges for developing feasible ETVG technology still linger and certainly are shared with many other areas of tissue engineering: (1) determining the appropriate/necessary functional requirements; (2) finding how to engineer (either *in vitro* or *in vivo*) an implant that meets them; and (3) predicting its performance after *in vivo* implantation (Butler et al., 2000). Several properties for viable clinical implantation are widely agreed upon, e.g., sufficient burst and fatigue strengths, stable and non-thrombogenic endothelium, and manufacturing feasibility and consistency. The 2nd challenge, "to

engineer a viable construct," is the *"Holy Grail"* of vascular tissue engineering (Conte, 1998). The answer to the question "How to make them?" is unknown. A multitude of different techniques do exist, yet most importantly, a reliable methodology to systematize results and compare them is non-trivial. The 3rd challenge, the prediction of *in vivo* performance, is complex, and knowledge is even more limited. The role of biomechanics regulating endothelial cells (for non-thrombogenicity), VSMCs (for vasoactivity), and fibroblasts (for ECM synthesis and maintenance) is all well-known at the cellular level; however, the causes for problems occurring at the implant-level that lead to graft failure date back to the earliest experiences with synthetic grafts and are still poorly understood. Short-term failure occurs due to thrombosis and endothelialization is influenced by graft dimensions and surface characteristics. Long-term failure occurs by intimal hyperplasia, specifically pannus-tissue borne from the native lumen at the anastomotic site, with proliferation and migration of VSMCs and FBs and synthesis of new ECM material. Causes for failure are multifactorial, poorly understood, but strongly associated with: (1) the mechanics of the compliance mismatch between the graft and native vessel (Greenwald and Berry, 2000), and (2) the biology of the shift from the acute inflammatory FBR toward homeostatic-driven long-term G&R (Szafron et al., 2018; Khosravi et al., 2020).

Critically designed, highly-controllable, computational-experimental paired approaches (in silico and *in vitro* and *in vivo*) are central to better inform, support, and validate rational frameworks describing and predicting bio-chemo-mechanical phenomena relevant to ETVG growth, development, and integration. Certainly, the intricacy of the inflammatory FBR to implanted engineered tissues or the relevant cross-talk between blood circulating cells/signaling factors and the blood vessel wall and its ECs,SMCs, and fibroblasts can only be studied with *in vivo* models. However, the translation of knowledge obtained *in vitro* at the cellular- and tissue-scales into more complex situations such as with tubular constructs stimulated by multi-axial modes of deformations, or due to the biological complexity of the *in vivo* milieu and the inflammatory FBR, is difficult to achieve with experiments alone. Better in silico models of engineered tissues, their manufacturing process, and their function post-implantation, based on physical, mechanical, and biochemical laws and systematic experimental data, will amongst others: (1) help to identify key regulating parameters, (2) extrapolate to predict behavior, and ultimately (3) minimize variability, increase quality, and optimize the design process (Geris, 2013).

Declaration of Competing Interest

None.

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