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Concise Review: The Use of Adipose-Derived Stromal Vascular Fraction Cells and Platelet Rich Plasma in Regenerative Plastic Surgery

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ABSTRACT

Tissue engineering has emerged at the intersection of numerous disciplines to meet a global clinical need for technologies to promote the regeneration of tissues. Recently, many authors have focused their attention on mesenchymal stem/stromal cells (MSCs) for their capacity to differentiate into many cell lineages. The most widely studied cell types are bone marrow mesenchymal stem cells and adipose-derived stem cells (ASCs), which display similar results. Biomaterials, cells, and growth factors are needed to design a regenerative plastic surgery approach in the treatment of organ and tissue defects, but not all tissues are created equal. The aim of this article is to describe the advances in tissue engineering through the use of ASCs, platelet rich plasma, and biomaterials to enable regeneration of damaged complex tissue. STEM CELLS *2016; 00:000–000*

SIGNIFICANCE STATEMENT

The aim of this article is to describe the advances in tissue engineering through the use of stromal vascular fraction, platelet rich plasma and biomaterials to enable regeneration of damaged complex tissue.

INTRODUCTION

A tremendous clinical need exists for the development of technologies to facilitate the regeneration of injured or diseased tissues. The unrelenting prevalence of trauma, congenital defects, and diseases drives the demand. A wide variety of tissues would benefit from engineering-based repair or regeneration, such as musculoskeletal tissues, bone, cartilage and soft tissues, including sub-cutaneous fat and skin. The field of tissue engineering is at the interface between bioengineering, materials science, biology, medicine, and surgery poised to meet these unmet clinical needs through the development of new technologies and refinement of existing ones. The increase of complexity in the targeted tissue for repair generally necessitate a concomitant increase in the complexity of the associated tissue engineering approach. Regardless of the complexity of the targeted tissue, bioengineering strategies generally involve the combined application of biomaterials, cells, and biologically active factors to promote new tissue formation. This process can involve de novo growth by in vitro and ex vivo culture or by in vivo regeneration. In recent years, many scientists have shown the existence of cells in the adult body that are capable of repairing and regenerating damaged tissues. In particular, adipose tissue is a multifunctional organ that contains various cellular types, such as mature adipocytes and the stromal vascular fraction (SVF) [1]. The SVF provides a rich source of adipose-derived stem cells (ASCs) that can be easily isolated from human adipose tissue and represent a viable alternative to bone marrow mesenchymal stem cells (BM-MSCs) [2]. Moreover, these cells can be added onto scaffolds, such as purified adipose tissue or biomaterials, which stimulate the long-term cell retention and subsequent colonization [3]. These techniques can be performed to treat hard tissue defects, such as bone and cartilage injuries, or soft tissue defects, such as scars and burn injury, and to regenerate various damaged tissues. In addition, the use of autologous growth factors derived from blood platelets can represent an effective support in tissue

regeneration for their ability to stimulate cell proliferation, differentiation and neoangiogenesis, favoring the wound healing process [4–6]. The well-known platelet-rich plasma (PRP) is a concentration of platelets in a small volume of plasma, that contains at least six major growth factors, including platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and transforming growth factor-β (TGF-β), released after platelet activation [5]. Recently, some authors such as Tobita et al. [8] and Jeong et al. [9] focused their attention on the beneficial use of ASCs and PRP in skin regeneration.

In this review we discussed, starting from our experience, gained over the last 10 years, the recent advances of tissue engineering in the wide field of regenerative plastic surgery using SVF/ASCs, PRP and new biomaterials, reporting the most innovative results achieved, and the possible drawbacks or risks associated with the clinical translation of these technologies.

Biomaterials and Nanotechnology: Bioengineered Scaffolds for Tissue Regeneration

Tissue engineering focuses its vocation on developing functional surrogates for damaged tissues and organs. Cells in the natural microenvironment are surrounded by the extracellular matrix (ECM), a complex three-dimensional (3D) structure composed of fibrous molecules, which forms the physiological scaffold supporting cell behavior [10].

Therefore, it is important that biomaterials, using for bioengineering scaffolds, can provide a 3D structure to support tissue growth. These scaffolds define and maintain the space in which the target tissue will form and can be tailored to support the attachment and proliferation of cells for tissue regeneration [11]. Ideally, a scaffold should serve as a transient structure that will degrade or resorb with time for tissue replacement. Advances in biomaterials science combined with increasing knowledge of ECM biology, and the role of environmental factors in tissue formation, have led to the development of scaffolds tailored to provide appropriate structural support and, in some cases, biological and mechanical cues to promote tissue regeneration in vivo [12–15]. Moreover, biomaterials for scaffolds can be modified to present biologically active signals, including cell-adhesion peptides and growth factors, favoring cell attachment and tissue formation [16–18]. Tissue engineering scaffolds seek to mimic key elements of the ECM and local microenvironment to support and induce tissue formation.

Naturally derived polymeric materials, including polypeptides and polysaccharides, such as collagen and hyaluronic acid [19, 20], have been extensively explored in the development of tissue engineered scaffolds for soft tissue repair. Indeed, a key advantage associated with naturally derived polymers is the general capacity of these materials to support cell attachment, proliferation, and differentiation [21, 22]. Although naturally derived polymers are enzymatically degradable, the kinetics of degradation may not be easily controlled or predicted. The generally weak mechanical strength associated with naturally derived polymers is also a limitation, but it may be improved through the introduction of intermolecular cross-links [23].

Synthetic polymers present several key advantages relative to naturally derived polymers. Synthetic polymers can be reproducibly manufactured with a wide range of mechanical properties and degradation kinetics to enable the production of scaffolds with properties tailored for a particular application [24]. For example, scaffolds composed of poly(lactic-co-glycolic acid) (PLGA) have been investigated for the regeneration of different tissues such as vascular network, bone and adipose tissue [25]. Many synthetic polymers undergo hydrolytic degradation, which may be more readily predicted and controlled than in vivo enzymatic degradation. Materials derived from the native ECM have also been explored as scaffolds. Acellular tissue matrices have been shown to support the ingrowth of tissues in several applications, without inducing a gross immune response [26, 27]. Indeed, given the natural origin of the matrices, they degrade slowly after implantation and are replaced or remodeled by the ECM derived from cells [28]. Decellularized matrices may also be processed to form particulates that can be used either alone or in combination with other materials to promote tissue repair [29]. As discussed above, apart from being 3D, the scaffold should be made from a biodegradable nontoxic material, capable of resorbing as a function of time to create space for new tissues, and should be highly porous to allow the diffusion of nutrients, oxygen, waste products and the interaction between cells and the microenvironment [30]. In fact, it is well known that the interplay between the cell and the surrounding ECM, at nanoscale levels, can rearrange cytoskeleton and induce specific cellular signaling for proliferation and differentiation [30]. So, nanotechnology can help tissue engineering to create nanostructured scaffolds that resemble the specific microenvironment [30]. In fact, cellular component structures and associated microenvironment show different structural and functional features according to their anatomical localization, ranging from nanometer to micrometer scale [10]. For this aim, many fabrication techniques have been developed to enable the making of 3D scaffolds with an interconnected porosity, ranging from particulate leaching techniques to electrospinning methods [11]. However, scaffold materials alone often lack the biological cues to induce tissue formation. Accordingly, scaffolds are commonly shaped for the release or controlled delivery of biologically active factors to induce tissue regeneration. In fact, the controlled delivery of biomolecules, such as growth factors and cytokines, is crucial to support and favor tissue regeneration [31]. So, it is important to understand the mechanisms of spatiotemporal regulation of the mechanotransduction pathways involved in cell-matrix interactions to improve the properties of biomimetic scaffolds, making possible the release of specific bioactive molecules to favor, in a controlled manner, cell proliferation and differentiation in vivo [30]. In literature, several studies have been reported about the regeneration capabilities of the combined use of scaffolds, growth factors and stem cells in vitro and in vivo. As reported by Scioli et al. [32], chondrogenesis and osteogenesis occurred spontaneously in human ASCs seeded into type I collagen gel scaffolds. Moreover, the use of PRP in combination with insulin enhanced ASC differentiation potential. In addition, Chung et al. [33] demonstrated that PEGylated fibrin gel was a good

ASC-carrying scaffold for encouraging local angiogenesis. Rat ASCs seeded into a gelatin-nanohydroxyapatite fibrous scaffold, in which PRP was previously incorporated, showed a stronger osteogenic and endothelial differentiation compared with scaffold without PRP [34]. Bayati et al. [35] demonstrated that ASCs seeded on electrospun polycaprolactone fibers favored keratinocyte differentiation in vitro and wound healing in vivo. Dua et al. [36] reported the esophageal regeneration, in human, using a metal stent covered by a commercially available ECM and sprayed with autologous PRP adhesive gel. In addition, Houdek et al. [37] demonstrated dermal regeneration using type I collagen, as a scaffold, in combination with PRP in a rat model of wound healing. For osteoarticular and soft tissue regeneration, β-tricalcium phosphate was proven an ideal scaffold. In fact, β-tricalcium phosphate scaffold in combination with PRP was reported to significantly induce 3D soft tissue augmentation in the cheek of mice 8 weeks after implantation [38]. In addition, the combination with PRP and BM-MSCs, seeded into a β-tricalcium phosphate scaffold, showed de novo formation of osteoarticular tissue in a canine model of articular cartilage defects [39], as well as ASCs, seeded into a porous β-tricalcium phosphate scaffold, that increased bony-union and mandibular body shape in a rabbit model of mandibulofacial defects [40].

Stem Cells in Tissue Regeneration

Scaffolds used in tissue engineering approaches are commonly divided into two general categories, namely, acellular scaffolds, which depend on cells in the recipient to effect tissue formation, and cellular scaffolds, which serve as cell transplantation vehicles. In both cases, the success of a scaffold technology toward achieving tissue growth depends largely on the action of the cells. Accordingly, many current efforts in tissue engineering seek to identify and optimize cell populations that can be leveraged for delivery with a scaffold to promote tissue repair where it otherwise might not occur. Autologous cell populations have been of great interest for applications in tissue engineering because of the minimal risk of rejection. Some early efforts in the field focused on isolating primary cells from a biopsy of the tissue of interest and growing the cells ex vivo for subsequent introduction back into the patient. Recently, Trovato et al. [41], reported the efficacy of a medical device called Rigeneracons® (CE certified Class I, Human Brain Wave Srl, Turin, Italy; www.hbwsrl.com) to provide autologous micrografts enriched of progenitor cells immediately available to be used in the clinical practice. The micrografts were obtained by the disaggregation of a small piece of tissue (e.g. periosteum, cardiac atrial appendage biopsy, and lateral rectus muscle of eyeball) followed by the selection of cell populations with a 50 micron strainer. These isolated progenitor cells showed high viability. However, major limitations encountered in this area are the difficulty in expanding cells to a sufficient number for clinical application, the necessity to do this expansion in Good Manufacturing Practices laboratories and to maintain the viability of these expanded cells [42]. As concerning ex vivo expansion, it has been reported that human ASCs, as well as BM-MSCs, after prolonged culture in vitro (prolonged passage for >4 months), are capable to undergo malignant transformation and to form tumors in immunodeficient mice [43, 44]. So, these findings indicate that long-term experiments in preclinical animal models will be further required and caution should be exercised in the ex vivo expansion of stem cells, including for example safety testing (e.g. karyotype analysis and tumorigenesis assays). In literature, many preclinical studies on animal models have been extensively reported for a wide variety of human pathologies using also different human stem cell sources (Table 1). While preclinical studies using animal models of Parkinson's disease, myocardial infarction or diabetes demonstrated that stem cells can regenerate damaged tissues and ameliorate disease symptoms, there are important factors that currently limit their clinical usage. For example, pluripotent human embryonic stem cells have been reported to form teratomas upon transplantation into immune compromised mice [45]; in addition, immune responses may elicit upon allogeneic transplantation [45]. Apart from the important clinical results obtained by autologous transplantation of human BM-MSCs and SVF/ASCs (Table 1) in soft tissue defect repair [46, 47], chronic wound healing [48, 49], myocardial regeneration [50, 51], spinal cord injury repair [52], neural regeneration in amyotrophic lateral sclerosis [53], Parkinson's disease [54], bone and cartilage regeneration [55, 56], encouraging results has been also reported with other stem cells, such as human fetal stem cells from umbilical cord and fetal annexes (Table 1). Umbilical cord (UC) and umbilical cord blood (UCB) represent a potential source of stem cells [57]. UC contains a mixture of stem and progenitor cells at different lineage commitment stages and UC was reported to be a good candidate for cell-based therapies and tissue engineering applications because of extensive self-renewal and multi-lineage differentiation potential of these stem cells [57]. The first isolated fetal stem cells were hematopoietic, derived from human UCB [57]. Thus, UCB represents the prototypical fetal stem cell source [57]. UCB cell population is a heterogeneous mixture of stem and progenitor cells, including mononuclear cells and mesenchymal stem cells (UCB-MSCs) [58]. UCB-MSCs have several advantages over the other stem cell sources. These MSCs showed similar cellular, morphological and differentiation properties compared with BM-MSCs but, at the same time, demonstrated advantages over BM-MSCs, whose number and differentiation potential decreased with age [57]. Fetal stem cell populations represent an abundant and accessible source of stem cells for research and clinical applications, including transplantations [59]. Many preclinical and clinical studies have been performed testing the regenerative properties of human fetal stem cells in different human pathologies, such as myocardial regeneration and neovascularization, neural and myogenic regeneration, lung injury repair, pancreatic beta cell regeneration, wound healing, bone and cartilage regeneration (Table 1).

ASC Isolation and Characterization

Although advances in cell culture protocols have allowed to expand autologous cells and to reach a sufficient number for clinical application, in some cases, diseased tissues or organs may not yield a sufficient number of autologous cells for tissue regeneration. As a result, tissue engineers seek to leverage autologous stem and progenitor cell populations, such as

Table 1. Principal human stem cell used in tissue regeneration: Preclinical and clinical studies

Stem cells	Preclinical study	Clinical study
Human bone marrow mesenchymal stem/stromal cells	• Wound healing and soft tissue defect repair (Arno AL et al. 2014; Markowicz M et al. 2006) • Cartilage and bone regeneration (Nakagawa Y et al. 2016; Shim JH et al. 2016; Yin W et al. 2016; Brennan MÁ et al. 2014) • Neural tissue regeneration in Alzheimer's disease (Liu Z et al. 2015; Shin JY et al. 2014; Bae JS et al. 2013), Parkinson's disease (Suzuki S et al. 2015; Xiong N et al. 2013) and post-cerebral ischemic stroke revascularization (Oh SH et al. 2015; Heo JS et al. 2013) • Myocardial regeneration (Bian S et al. 2014; Armiñán A et al. 2010; Williams AR et al. 2013) • Hepatic regeneration (Manzini BM et al. 2015) • Lung epithelium repair (Fang X et al. 2015) • Pancreatic beta cell regeneration (Gabr MM et al. 2015)	• Lung injury repair in acute respiratory distress syndrome (Wilson JG et al. 2015, phase 1, NCT01775774) • Soft tissue defect repair (Xie HQ E et al. 2014; Jianhui Z et al. 2014) • Chronic wound healing (Wettstein R et al. 2014; Falanga V et al. 2007) • Myocardial regeneration (Karantalis V et al. 2014, PROMETHEUS phase 1/2, NCT00587990) and post-ischemic neovascularization (Li M et al. 2013) • Spinal cord injury repair (Mendonça MV etal. 2014, phase 1, NCT01325103) • Immunomodulatory effects and neural regeneration in multiple sclerosis and amyotrophic lateral sclerosis (Mazzini et al. 2010, phase 1, n. 16454-pre21-823; Karussis D et al. 2010, phase 1, NCT00781872) and Parkinson's disease (Venkataramana NK et al. 2012) • Bone and cartilage regeneration (Centeno CJ et al. 2010; Koga et al. 2008)
Human stromal vascular fraction/ adipose-derived stem/stromal cells	• Cartilage and bone regeneration (Toupet K et al. 2015; Zorzi AR et al. 2015; Wang W et al. 2015) • Corneal epithelium repair (Alio del Barrio JL et al. 2015) • Myogenic regeneration in Duchenne muscular dystrophy (Pelatti MV et al. 2016) • Immunomodulatory and anti-inflammatory effects in acute graft-versus-host disease (Kim KW et al. 2015), systemic lupus erythematosus (Choi EW et al. 2016), autoimmune arthritis (Yi H et al. 2015)and rheumatoid arthritis (Choi EW et al. 2016) • Myogenic regeneration in urological dysfunction (Yiou R et al. 2016) • Hepatic regeneration (Manzini BM et al. 2015) • Wound healing (Park IS et al. 2015; Souza CM et al. 2014) • Neural regeneration in Parkinson's disease (Schwerk A et al. 2015), Alzheimer's disease (Chang KA et al. 2014; Ha S et al. 2014) and peripheral nerve regeneration (Lasso JM et al. 2015) • Soft tissue defect repair (Xu FT et al. 2016; Zhang Q et al. 2015; Cheung HK et al. 2014) • Myocardial regeneration (Savi M et al. 2015)	• Soft tissue defect repair (Tanikawa DY et al. 2013; Gentile P et al. 2012; Jo DI et al. 2013; Rigotti G et al. 2016; Kølle SF et al. 2013; Matsumoto D et al. 2006) • Hard-tissue defect repair (Sándor GK et al. 2014, R03058; Lendeckel S et al. 2004) • Neovascularization in systemic sclerosis (Granel B et al. 2015, NCT01813279, phase 1) • Chronic wound healing (Cervelli V et al. 2011) • Bone and cartilage regeneration (Koh YG et al. 2016; Jo CH et al. 2014; Pak J et al. 2011) • Myogenic regeneration and neovascularization in erectile dysfunction (Haahr MK et al. 2016, NCT02240823, phase 1) • Pancreatic beta cell regeneration and immunomodulatory effects in type 1 diabetes mellitus (Thakkar UG et al. 2015) • Crohn's fistula repair (Cho YB et al. 2015, phase 2; García-Olmo D et al. 2005, phase 1) • Myocardial regeneration in post- ischemic cardiomyopathy (Perin EC et al. 2014, PRECISE trial, NCT00426868, phase 1) • Angiogenesis in nonrevascularizable limb ischemia (Bura A et al. 2014, NCT01211028, phase 1/2) • Lung alveolar epithelium repair in acute respiratory distress syndrome (Zheng G et al. 2014, NCT01902082, phase 1) • Retinal regeneration in age-related macular degeneration (Limoli PG et al. 2014)
Human fetal stem cells: • Umbilical cord mesenchymal stem cells • Umbilical cord blood unrestricted somatic stem cells • Umbilical cord blood mononuclear cells • Umbilical cord perivascular cells • Umbilical cord Wharton's jelly • Amnion epithelial cells	• Bone and cartilage regeneration (Diao Y et al. 2009; Heidari S et al. 2013) • Tendon regeneration (Emrani H et al. 2011) • Myocardial regeneration (Cortes-Morichetti M et al. 2007; Weiss ML et al. 2013) • Hepatic regeneration (Tang Y et al. 2016; Shi D et al. 2016) • Immunomodulatory effects in rheumatoid arthritis (Greis S et al. 2012; Liu Y et al. 2010) • Neovascularization in ischemic limb disease and Buerger's disease (Weiss ML et al. 2013) • Neural regeneration in spastic paresis (Meier C et al. 2006), spinal cord injury (Sankar V et al. 2003), amyotrophic lateral sclerosis (Garbuza-Davis S et al. 2003), Parkinson's disease (Xiong N et al. 2010) and Alzheimer's disease (Lee J et al. 2012) • Wound healing (Liu L et al. 2014; Liao Y et al. 2013; Zebardast N et al. 2010) and epithelial regeneration (Dai Y et al. 2007; Zhang Y H et al. 2012)	• Immunomodulatory and anti-inflammatory effects in severe atopic dermatitis (Kim HS et al. 2016, phase 1/2a) • Myocardial regeneration and neovascularization in chronic ischemic cardiomyopathy (Can A et al. 2015, NCT02323477, phase 1/2; Zhao XF et al. 2015) and hypoplastic left heart syndrome (Burkhart HM et al. 2015) • Neural regeneration in spinal cord injury, cerebral palsy, post-traumatic brain syndrome, post-brain infarction syndrome, spinocerebellar ataxias, and motor neuron disease (Miao X et al. 2015; Wang X et al. 2015; Kang KS et al. 2005) • Immunomodulatory and regenerative effects in type I diabetes (Cai J et al. 2016, NCT01374854, phase 1/2) and type II diabetes (Kong D et al. 2014) • Immunomodulatory and regenerative effects in severe and treatment-refractory systemic lupus

Table 1. *Continued*

Stem cells	Preclinical study	Clinical study
• Amnion mesenchymal stromal cells • Placental mesenchymal stem cells	• Pancreatic beta cell regeneration (Ende N et al. 2004) • Acute kidney injury repair (Večerić-Haler Ž et al. 2016)	erythematosus (Wang D et al. 2014, NCT01741857, phase 1/2) and lupus nephritis (Gu F et al. 2014) • Myogenic regeneration in Duchenne Muscular Dystrophy (Rajput BS et al. 2015) • Immunomodulatory and regenerative effects in multiple sclerosis (Li JFet al. 2014) • Lung injury repair in bronchopulmonary dysplasia (Chang YS et al. 2014, NCT01297205, phase 1) • Hematopoietic recovery in hematological malignancies (Wagner JE Jr et al. 2014, NCT00412360, phase 3)
Human embryonic stem cells	• Myocardial regeneration (Laflamme MA et al. 2007) • Pancreatic beta cell regeneration (Hua XF et al. 2014) • Crohn's fistula repair (Ferrer L et al. 2016) • Retinal regeneration (Brant Fernandes RA et al. 2016) • Neural regeneration in Huntington disease (Vazey EM et al. 2010), Parkinson's disease (Samata B et al. 2015) and Alzheimer's disease (Yue W et al. 2015) • Kidney injury repair (van Koppen A PLoS One et al. 2012) • Hepatic regeneration (Woo DH et al. 2012) • Wound healing (Lee MJ et al. 2011) • Bone regeneration (Arpornmaeklong P et al. 2010) • Cartilage regeneration of vocal fold and articular regeneration (Cedervall J et al. 2007; Zhang S et al. 2013)	• Retinal regeneration in age-related macular degeneration and Stargardt's disease (Song WK et al. 2015; Schwartz SD et al. 2015, in collaboration with Ocata Therapeutics MA09-hRPE NCT02122159) • Neural regeneration in severe spinal cord injuries (Geron FIH trial 2010GRNOP1; Asterias Biotherapeutics AST-OPC1, NCT02302157)
Human hematopoietic stem cells	• Myocardial regeneration (Armiñán A et al. 2010) • Bone regeneration (Wagner F et al. 2016) • Wound healing (Costantini TW et al. 2016) • Hematopoietic recovery in hematological malignancies(Serra-Hassoun M et al. 2014; Abe T et al. 2014)	• Myocardial regeneration and revascularization (Ince H et al. 2005, FIRSTLINE-AMI trial; Seiler C et al. 2001) • Post-limb ischemic revascularization (Kawamoto A et al. 2009, NCT00221143, phase 1/2a) • Hematopoietic recovery in hematological malignancies (Jagirdar N et al. 2015; Danylesko I et al. 2016; Grosicki S et al. 2015) • Hepatic regeneration (Zekri AR et al. 2015; King A et al. 2015) • Multiple sclerosis (Mancardi GL et al. 2015) • Chronic spinal cord injuries (Al-Zoubi A et al. 2014) • Bone regeneration (Marx RE et al. 2014; Kuroda R et al. 2014) • Pancreatic beta cell regeneration and immunomodulatory effects in Type 1 diabetes mellitus (Thakkar UG et al. 2015)
Human peripheral blood mononuclear cells	• Osteochondral defects (Hopper N et al. 2015) • Neural regeneration (Haider T et al. 2015) • Neovascularization (Kang J et al. 2014; Kim SW et al. 2010) • Bone regeneration (Chim H et al. 2006) • Myocardial regeneration (Kang J et al. 2014; Barclay GR et al. 2012)	• Myocardial regeneration(Delewi R et al. 2015; Perin et al. 2004)and neovascularization (van der Laan AM et al. 2011; Hirsch A et al. 2011 • Bone marrow replacement: hematological malignancies • (Mielcarek M et al. 2012) • Wound healing and neovascularization (De Angelis B et al. 2014) • Cerebral injury repair and revascularization (Chen DC et al. 2014, NCT00950521, phase 2)
Human induced pluripotent stem cells	• Neural regeneration in Parkinson's disease (Kikuchi T et al. 2015; Sundberg M et al. 2013; Kriks S et al. 2011), spinal cord injury (Cummings BJ et al. 2005), and Alzheimer's disease (Yue W et al. 2015; Fujiwara N et al. 2015) • Myogenic regeneration (Choi IY et al. 2016) • Bone and ligament regeneration (Kouroupis D et al. 2016) • Integumentary organ system repair (Takagi R et al. 2016) • Pancreatic beta cell regeneration (El Khatib MM et al. 2016)	• Retinal regeneration: age-related macular degeneration(Takahashi M, RIKEN clinical trial 2014)

Table 1. *Continued*

Stem cells	Preclinical study	Clinical study
	• Smooth muscle regeneration (Wang Z et al. 2016) • Myocardial regeneration (Wendel JS et al. 2015; Ye L et al. 2014) and neovascularization (Zhang H et al. 2015) • Hepatic regeneration (Ramanathan R et al. 2015; Chien Y et al. 2015; Carpentier A et al. 2014) • Kidney injury repair (Imberti B et al. 2015) • Skin regeneration in recessive dystrophic epidermolysis bullosa (Sebastiano V et al. 2014) • Respiratory epithelium regeneration (Huang SX et al. 2014)	

BM-MSCs and ASCs [60]. The latter can be isolated from visceral and subcutaneous adult adipose tissue by enzymatic digestion [43]. After centrifugation, a heterogeneous cell population named stromal-vascular fraction (SVF) is obtained, consisting of endothelial cells, smooth muscle cells, fibroblasts, pericytes, mast cells, T cells, preadipocytes and ASCs [43]. After SVF plating, the adherent replicative cells are conventionally termed adipose tissue-derived stem cells (ASCs) and are able to differentiate in multiple cell lineages [43]. Adipose tissue is an abundant and easily accessible source for adult stem cell harvesting. Before discovering of the plasticity of ASCs, bone marrow was clinically considered the major source of human adult stem cells, the so-called mesenchymal stem/stromal cells (MSCs) [61]. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has proposed a minimal set of four criteria to define human mesenchymal stem cells (MSCs) [62]:

1. MSCs are plastic-adherent when maintained under standard culture conditions.
2. MSCs have the capability to differentiate in vitro in osteoblasts, adipocytes, chondroblasts (as demonstrated by staining of in vitro cell culture) [62].
3. MSCs express CD73, CD90, and CD105.
4. MSCs lack expression of the hematopoietic lineage markers c-kit, CD14, CD11b, CD34, CD45, CD19, CD79, and human leukocyte antigen-DR.

The ASCs meet the majority of the ISCT's criteria for MSCs; however, Lin et al. [63] have found that ASCs exist as $CD34^+$ $CD31^-$ $CD104b^-$ α-SMA^- cells in the capillaries and in the tunica adventitia of larger vessels in vivo. In the capillaries, these cells coexist with pericytes and endothelial cells, both of which are possibly related to ASCs. In the tunica adventitia of larger vessels, these ASCs exist as specialized fibroblasts (having stem cell properties). Corselli et al. [64] previously demonstrated that human pericytes, which encircle capillaries and microvessels, give rise in culture to genuine MSCs. This raised the question of whether all MSCs derive from pericytes. Pericytes and other cells defined on differential expression of CD34, CD31, and CD146 were sorted from the SVF of human white adipose tissue. Besides pericytes, $CD34^+$ $CD31^-$ $CD146^-$ $CD45^-$ cells, which reside in the outmost layer of blood vessels, the tunica adventitia, natively express MSC markers and give rise in culture to clonogenic multipotent progenitors identical to standard BM-MSCs. Despite common MSC features and developmental properties, adventitial cells and pericytes retain distinct phenotypes and genotypes through culture. However, in the presence of growth factors involved in vascular remodeling, adventitial cells acquire a pericyte-like phenotype. In conclusion, Corselli et al. [64] demonstrated the coexistence of two separate perivascular MSC progenitors: pericytes in capillaries and microvessels and adventitial cells around larger vessels.

Factors such as donor age, adipose tissue type (white or brown) and anatomical location (subcutaneous or visceral adipose tissue), type of surgical procedure, culture conditions, exposure to plastic, plating density, and medium formulations might influence the proliferation rate as well as the differentiation potential of these cells. For example, peroxisome proliferator-activated receptor-γ (PPAR-γ) (an indicator of adipogenic potential and commitment) is more expressed in the adipose tissue of the arm [65]. In addition, there is evidence that the ability of adipose precursors to grow and differentiate varies among different fat depots and changes with age [65]. Younger patients have increased PPAR-γ-2 expression in all depots, whereas older patients have a consistent expression only in the arm and thigh depots [65]. In fact, the growth rate of ASCs was higher in younger (25-30 years old) than in older patients. Abdominal subcutaneous adipose tissue appears to be less susceptible to apoptotic stimuli [65]. Levi et al. [66] reported that subcutaneous fat depots retain markedly different osteogenic and adipogenic potentials. Osteogenesis is most robust in ASCs from the flank and thigh, as compared with those from the arm and abdomen. This is accompanied by elevations in bone morphogenetic protein 4 and bone morphogenetic protein receptor 1B [66]. The osteogenic advantage of cells from the flank and thigh is also observed when the paracrine effects of these cells are analyzed [66]. Conversely, those cells isolated from the flank have a lesser ability to undergo adipogenic differentiation. Adipose-associated HOX genes are less expressed in flank-derived ASCs [66]. Variations exist between fat depots in terms of ASC osteogenic and adipogenic differentiation. Differences in HOX expression and bone morphogenetic protein signaling may underlie these observations [66]. This study indicates that the choice of fat depot for ASC isolation may be a pivotal factor for future efforts in tissue engineering. Neither the type of surgical procedure nor the anatomical site of the adipose tissue affects the total number of viable cells that can be obtained from the SVF [67]. However, since different anatomical localizations of fat tissue have their own metabolic characteristics, such as lipolytic activity, fatty acid composition, and

gene expression profile, the source of subcutaneous adipose tissue (abdominal subcutaneous versus peripheral subcutaneous fat) might influence the long-term characteristics of the fat graft [67]. In a study by Aksu et al. [68], human ASCs were isolated from superficial or deep adipose layers of the abdominoplasty specimens obtained from patients (male and female) undergoing elective surgeries. In the female, ASC osteogenic differentiation did not differ between superficial or deep adipose layers. In the male, ASCs from superficial depots differentiated faster and more efficiently than those of the deep layers. Male ASCs, from both depots, differentiated more effectively than female ASCs from both adipose layers. The frequency of proliferating ASCs and the population doubling time are dependent on the surgical procedure, with some advantages for resection and tumescent liposuction compared with ultrasound-assisted liposuction [67]. In one study comparing BM-MSCs and ASCs [69] from the same patient, no significant differences were observed in the yield of adherent stromal cells, growth kinetics, cell senescence, multilineage differentiation capacity, or gene transduction efficiency. Metabolic characteristics and fat cell viability seem not to differ when comparing standard liposuction with syringe aspiration and no unique combination of preparation or harvesting techniques has been shown superior to date [67]. Although attachment and proliferation capacity are more pronounced in ASCs derived from younger donors compared with older donors, the differentiation capacity is maintained with aging [67]. ASCs have the same differentiation potential as described for BM-MSCs. However, some characteristics, such as the colony-forming unit frequency and the maintenance of proliferating ability in culture, seem even to be superior in ASCs compared with BM-MSCs [67]. This is a significant advantage of ASCs, considering that the quality of BM-MSCs in general is compromised when exposed to long-term culture [70]. In particular prolonged culture of BM-MSCs has been shown to limit differentiation potential and proliferative capacity. Furthermore, BM-MSCs undergo senescence with prolonged in vitro culture, a phenomenon that has not been observed in ASCs [70]. According to Cervelli et al. [4], SVF manual extraction was performed as following: liposuction aspirate was washed three times with phosphate-buffered saline (PBS) and suspended in an equal volume of PBS and 0.1% collagenase type I (C130; Sigma-Aldrich, Milan, Italy, http://www.sigmaaldrich.com) prewarmed at 37°C. Then adipose tissue was placed in a shaking water bath at 37°C with continuous agitation for 60 minutes and centrifuged at 600*g* for 10 minutes at room temperature. The supernatant, containing mature adipocytes, was discarded. Then the SVF pellet was resuspended in erythrocyte lysis buffer (155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM ethylenediaminetetraacetic acid [EDTA]) and incubated for 5 minutes at room temperature. After centrifugation at 600*g* for 5 minutes, the pellet was resuspended in few microliters of growth medium and passed through a 100-μm Falcon strainer (Becton and Dickinson, CA, US, http://www.bd.com), then cells were counted using a hemocytometer. Cell viability by trypan blue exclusion was consistently more than 98%. We obtained ~250,000 ± 34,782 nucleated cells per milliliter of adipose tissue ($n = 12$ donors) [47].

For automatic SVF extraction, it is possible to use or not enzymatic digestion. In the first case, Celution system (Cytori Therapeutics, Inc., CA, US, http://www.cytori.com) was used until to 2014 [63]. Briefly, adipose tissue was introduced into the cell-processing device and subsequently washed to remove red blood cells and debris, and then enzymatically digested with collagenase type I. Upon digestion of adipose tissue and release of mononuclear cells from the adipose tissue matrix, the released cells were transferred into the centrifuge processing vessel. Then, cells within the suspension were concentrated by short centrifugation and wash cycles. The cycles were repeated until the entire volume of input cell suspension was processed and cell population localized into the output chamber. The cells were washed one final time and then suspended for use in 5 mL of Ringer's lactate solution. An aliquote of this SVF solution was incubated in erythrocyte lysis buffer, and after centrifugation at 600*g* for 5 minutes, cell population was counted using hemocytometer. Cell viability by trypan blue exclusion was consistently more than 98%. The cell yield was ~50,000 ± 6,956 nucleated cells per mL of adipose tissue ($n = 12$ donors) [47]. In the second case, it is possible to isolate SVFs by mechanical filtration of the fat. Briefly, through the use of P.R.L. Platelet Rich Lipotransfert system (CORIOS Soc. Coop, Milan, Italy, http://www.corios.it), the fat (80 mL) was subjected to automatic filtration and centrifugation cycles at 1,100*g* for 10 minutes, after which 40 mL of the suspension was extracted from the bag. The suspension was further filtered through a 120-μm filter, obtaining about 20 mL of SVF suspension. The latter was centrifuged at 600*g* for 10 minutes and then pellet was resuspended in erythrocyte lysis buffer and incubated for 5 minutes at room temperature. After centrifugation at 600*g* for 5 minutes, the pellet was resuspended in few microliters of growth medium and cell population was counted using hemocytometer. Cell viability by trypan blue exclusion was consistently more than 98%. About 65,000 ± 3,345 nucleated cells per milliliter of fat tissue were obtained ($n = 12$ donors).

MSCs have received a great deal of attention in tissue engineering and advances with other adult stem cells have generally progressed slowly, in part due to difficulties associated with maintaining stem cells in culture or achieving attachment of these cells to scaffolds [71].

Platelet Rich Plasma and Growth Factors

The literature about the PRP is considerable, but the published results are often contradictory. It is very important to sort and interpret the available data, due to a large number of preparation techniques, terminologies, forms of these materials and the endless of potential applications.

It is possible to identify four main types of PRP preparation depending on cell content and fibrin architecture:

1. Pure Platelet-Rich Plasma or leukocyte-poor PRP products are preparations without leukocytes and with a low-density fibrin network after activation;
2. Leukocyte and PRP products are preparations with leukocytes and with a low-density fibrin network after activation (the largest number of commercial or experimental systems);
3. Pure Platelet-Rich Fibrin or leukocyte-poor platelet-rich fibrin preparations are preparations without leukocytes and with a high-density fibrin network.

4. Leukocyte and Platelet Rich Fibrin or second generation PRP products are preparations with leukocytes and with a high-density fibrin network.

Growth factors contained in PRP, including angiogenic factors, such as VEGF, and osteogenic factors, such as bone morphogenetic protein-2, have been used to promote tissue formation in soft tissue defects [49, 72, 73], periodontal defects [74, 75], oral surgery [76–78], maxillofacial surgery [79–81], aesthetic plastic surgery [82–84], spinal fusion [85–87], and heart bypass surgery [88].

Briefly, the process of preparing PRP consists of four phases: blood collection, centrifugation for platelet concentration, induction of gelation (if the PRP is to be used in gel form), and activation [4]. Current systems for the preparation of platelet concentrations routinely report the use of various centrifugation rates (Table 2). After centrifugation, the buffy coat layer, consisting of platelets and white blood cells, was sequestered in a small or large volume of plasma. Anitua et al. [89] reported the use of two centrifugation rates. Blood was collected into 3.8% (wt/vol) sodium citrate and centrifuged at 4,500*g* for 12 minutes at 4°C to obtain platelet poor plasma (PPP) or at 460*g* for 8 minutes to obtain PRP. Calcium chloride was added to PPP and PRP at a final concentration of 22.8 mM. The secretion of growth factor begins with platelet activation. We prepared PRP from a small volume of blood (18 ml) according to the method of the Cascade-Selphyl-Esforax system (Aesthetic Factors, LLC, NJ, US, http://www.selphyl.com) with some modifications, and from 60 ml of blood according to the P.R.L. Platelet Rich Lipotransfert system (CORIOS Soc. Coop, Milan, Italy, http://www.corios.it) with some modifications, using PRP alone (C-punt; Biomed Device, Modena, Italy, http://www.biomeddevice.it) without SVF cells. Briefly, blood was taken from a peripheral vein using sodium citrate as an anticoagulant. The current systems for preparing platelet concentrations use two centrifugation speeds (in the Cascade-Selphyl-Esforax procedure, we used 1,100*g* for 10 minutes; in the P.R.L. Platelet Rich Lipotransfert system, we used 1,000*g* for 10 minutes). PRP was prepared in all cases with approval of the transfusion service. Although the method of preparation is not selective and may include leukocytes, the final aim is to obtain a platelet pellet. Growth factors are secreted only once platelet activation begins, by adding calcium ($Ca2^{+}$). To optimize the secretion process, the optimum concentration of $Ca2^{+}$ was previously determined [4]. In the Cascade-Selphyl-Esforax system, autologous PRP (9 ml) obtained after centrifugation, and not yet activated, was switched to 10 ml tubes containing $Ca2^{+}$. Autologous PRP, not yet activated, obtained by the P.R.L. Platelet Rich Lipotransfert procedure after centrifugation (20 ml), was inserted in a light selector device. At the end of the procedure, 9 ml of PRP was harvested. There are many standard cell separators and salvage devices that can be used to produce PRP. These devices operate on a unit of blood and typically use continuous-flow centrifuge bowl or continuous-flow disk separation technology with a hard (fast) and a soft (slow) spin, yielding platelet concentrations of 2x to 4x baseline [90, 91]. Such devices include the CATS (Fresenius, DE, US, http://www.fmcna.com), Sequestra (Medtronic, MN, US, http://www.medtronic.com), Hemonetics Cell Saver 5 (Haemonetics Corp., MA, US, http://www.haemonetics.com), and others [90, 91]. However, many surgical procedures require the use of relatively small volumes of PRP [83]. Consequently, small, compact office systems have been developed to produce approximately 6 ml of PRP from 45-60 ml of blood, such as the GPS (Biomet, Warsaw, IN, http://www.biomet.com), PCCS (Implant Innovations, Inc., FL, US, http://www.3i-online.com), Symphony II (DePuy, IN, US, http://www.depuy.com), SmartPReP (Harvest Technologies Corp., MA, US, http://www.harvesttech.com), and Magellan (Medtronic, MN, US, http://www.medtronic.com) [83, 92, 93]. Although all operate on a small volume of drawn blood (45-60 ml) and on the principle of centrifugation, these systems differ widely in their ability to collect and concentrate platelets, with approximately 30%-85% of the available platelets collected and from a less than twofold to an approximately eightfold increase in the platelet concentration over baseline [90, 91].

There are also other systems for the PRP preparation, such as Fibrinet (Cascade Medical Enterprises, Plymouth, UK, http://www.cascademedical.com), Regen (Regen Lab, Lausanne, Switzerland, http://www.regenkit.com), Plateltex (Plateltex S.R.O., Bratislava, Slovakia, http://www.plateltex.com), and Vivostat (Vivostat A/S, Borupvang, Denmark, http://www.vivostat.com). We also prepared PRP according to the C-punt method (C-punt; Biomed Device, Modena, Italy, http://www.biomeddevice.it), and in all cases, the authors worked under a protocol approved by our institution's transfusion service according to Decree Law November 2, 2015.

In general, most systems, whether large or small volume, do not concentrate the plasma proteins of the coagulation cascade [90]. The concentration of plasma proteins above baseline can be achieved through secondary ultrafiltration, using for example the UltraConcentrator (Interpore Cross, CA, US, http://www.biomet.com) and the Access System (Interpore Cross), in which the buffy coat collected from a centrifugation stage is passed through hollow fibers with an effective pore size of 30 kDa. This system removes by filtration up to two-thirds of the aqueous phase; thus, the concentrations of the retained plasma proteins and formed elements are increased substantially. Mazzucco et al. described the different growth factor concentrations that are obtained through different devices (Fibrinet, Plateltex, and Regen) and a homemade method [94]. The PDGF-BB, TGF-β, and IGF-1 were detected in lower concentrations with the use of Fibrinet. In contrast, the Regen method showed high concentrations of TGF-β, b-FGF, and IGF-1, whereas the Plateltex method showed a high level of VEGF [94].

As discussed above, many different procedures to optimize the protocol for PRP preparation were reported in literature. Amable et al. [95] studied variations in relative centrifugal force (RCF or *g*-force), temperature, and time for optimizing conditions for platelet isolation and quantification of cytokines and growth factors in PRP before and after platelet activation. Peripheral blood from 22 healthy male and female volunteer donors (20-54 years old) was collected using 4.5 mL blood collection tubes containing 0.5 ml citrate solution (VacutainerW, Ref: 369714; BD Biosciences, CA, US, http://www.bd.com). Blood samples were run by choosing RCF from 240 to 400*g*, time from 8 to 19 minutes and temperature from 8° to 16°C. All steps were performed in a refrigerated centrifuge (certified Jouan Br4i, Nantes, France, https://www.thermofisher.com). The best performance was

Table 2. Platelet rich plasma collection systems

Study and procedures	Processed blood (ml)	Anti-coagulant	Centrifugation 1TH step (RCF —*g*-force)	Centrifugation 2thstep (RCF—*g*-force)	Activator	Platelet count rise
***Mazzucco* et al.** FIBRINET	7	ACD+ separator gel	1,100*g* × 10 minutes	1,500*g* x15min	Ca^{++}, High speed centrifugation	1358 ± 419 x10^3
***Mazzucco* et al.** PLATELTEX	6	Sodium citrate	180*g* × 10 minutes	1,000*g* × 10min	Ca^{++}, batroxobin	1160 ± 164 x10^3
***Mazzucco* et al.** REGEN	8	ACD + separator Gel	1,500*g* × 10 minutes	—	Autologous thrombin	430 ± 109 x10^3
***Gentile* et al.** Cascade-Selphyl-Esforax	9-18	ACD + separator Gel	1,100*g* × 10 minutes	1,500*g* x15 minutes	Ca^{++}, High speed centrifugation	8.8 x10^4 2 times
***Gentile* et al.** C-PUNT	60	ACD	260*g* × 10minutes	1,200*g* × 10 minutes	—	4.0 times PLASMA 553.1 ± 43.79 x10^6 PRP 925.9 ± 52.03 × 10^6
***Amable* et al.**	4.5	Sodium citrate	300*g* × 5 minutes	700*g* x17 minutes	—	1.4 × 10^6- 1.9 × 10^6 5.4-fold-7.3-fold
***Anitua* et al.** PRGF Endoret (BTI)	4.5	Sodium citrate + buffy coat	580*g* × 8 minutes	—	Ca^{++}	2.67 times
***Amanda* et al.**	3.5		100*g* × 10 minutes	400*g* × 10 minutes	—	1.222 ± 166 × 10^3 5-fold
***Kahn* et al.**	478	—	3731*g* × 4 minutes	—	—	8.3 × 10^{10}
Slichter and Harker	250-450	—	1000*g* × 9 minutes	3000*g* × 20 minutes	—	80%recovery
***Landesberg* et al.**	5	—	200*g* × 10 minutes	200*g* × 10 minutes	—	5.57- 9.35 × 10^8
***Jo* et al.**	9	—	900*g* × 5 minutes	1,500*g* × 15 minutes	—	633.2 ± 91.6 × 10^3 4.2 times
***Bausset* et al.**	8.5	—	250*g* × 15 minutes	250*g* × 15 minutes	—	3.96 times
***Mantalvo* et al.**	8.5	—	160*g* × 10 minutes	400*g* × 10 minutes	—	630.2 × 10^3
***Araki* et al.**	7.5	—	270*g* × 10 minutes	2,300*g* × 10 minutes	—	189.6 × 10^4
***Kececi* et al.**	9	—	250*g* × 10 minutes	750*g* × 10 minutes	—	679.9 × 10^3

Abbreviations: ACD, acid citrate dextrose; RCF, relative centrifugal force; —, no data.

obtained using parameters of 300*g* for 5 minutes at 12°C and 240*g* for 8 minutes at 16°C for the first spin. The second spin of 700*g* for 17 minutes was chosen since it allowed a lower platelet loss into the PPP fraction and produced a pellet that was easily resuspended [95]. Amanda et al. [96] demonstrated that the processing of 3.5 ml of blood at 100*g* for 10 minutes (1st spin), 400*g* for 10 minutes (second spin) and withdrawing 2/3 of remnant plasma, promoted high platelet recovery (70%-80%) and concentration (5×) maintaining platelet integrity and viability. Authors believe that time and acceleration are the fundamental parameters that define the composition of the PRP sample after the first spin step. Longer time periods slightly increased platelet recovery and decreased the concentrations of leukocytes in the upper layer. Therefore, time could be a control parameter when low levels of leukocytes, such as granulocytes and lymphocytes, are required in the PRP sample. Various factors contribute to platelet concentration gradient such as the size of platelets, the biological difference among individuals and hematocrit variability. However, this gradient is more critical after the second spin step because some erythrocytes are inevitably present in the volume that was transferred from the first spin. The presence of these remaining erythrocytes can generate a pellet at the bottom of the tube, which adsorbs platelets and leukocytes on its surface. The manual mixing for a short period of time is insufficient to completely resuspend the platelets, and a large variability in platelet counting is observed. Approximately 20% of platelets remained adsorbed in the erythrocyte pellet. Kahn et al. [97] determined that a centrifugal acceleration of 3,731*g* for a period of 4 minutes was the optimal condition for obtaining the highest platelet concentration from 478 ml of whole blood. The highest platelet recovery efficiency obtained by Slichter and Harker [98] was 80%, using a sample of 250-450 ml of whole blood centrifuged at 1,000*g* for a period of 9 minutes. It was observed that a subsequent centrifugation step of 3,000*g* for a period of 20 minutes decreased the platelet viability. Landesberg et al. [99] obtained PRP samples that had approximately 3.2 times the concentration of the whole blood baseline. The centrifugation procedure processed 5 ml of whole blood for two spins at 200*g* for 10 minutes per spin. Jo et al. [100] examined the effect of the centrifugation time and gravitational force (*g*) on the platelet recovery ratio of PRP. Two-step centrifugations for preparing PRP were used in 39 subjects. Whole blood was centrifuged from 500*g* to 1,900*g* at 200*g* increments for 5 minutes and from 100*g* to 1300*g* at 200*g* increments for 10 minutes. In the step 2, platelets in the separated plasma were concentrated at 1,000*g* for 15 minutes, 1,500*g* for 15 minutes, 2,000*g* for 5 minutes, and 3,000*g* for 5 minutes. They achieved better efficiency (92%) by applying an acceleration of 900*g* for 5 minutes for the first spin step. A total of 9 ml of WB were processed, and the platelet concentration was measured to be $310.7 \pm 78.5 \times 10^3/mm^3$. The maximum efficiency for the second spin step (84%) was obtained by applying 1,500*g* for 15 minutes. The platelet concentration was $633.2 \pm 91.6 \times 10^3/mm^3$, which was 4.2 times greater than the baseline concentration. Bausset et al. [101] found that a centrifugation of 130*g* or 250*g* for a period of 15 minutes was optimal when performing a procedure that involved two spins. A platelet concentration factor of 3.96 was obtained from the 8.5 ml WB processed, and 2 ml of plasma was processed in the second spin step. Montalvo et al. [102] compared two methods for obtaining PRP: Double centrifugation (ACE system; Surgical Supply and Surgical Science Systems, MA, US, https://www.acesurgical.com) and single centrifugation (Nahita System; Navarra, Spain, http://www.acegroup.lu). Three test tubes of 8.5 ml whole blood each were introduced into an ACE centrifuge machine and subjected to a force of 160*g* for 10 minutes. For the second centrifugation, 400*g* force for 10 minutes was applied. In the Nahita system, blood was extracted into 3.5-ml citrated tubes (Venojet; Terumo MR, Tokyo, Japan, https://www.terumotmp.com) containing 0.5 ml of trisodium citrate, citrate, and ACD as anticoagulants. Test tubes were centrifuged with a 280*g* for 7 minutes. Platelet concentration from the ACE and Nahita systems were (336%) and (227%), respectively. Mazzocca et al. [103] analyzed three protocols for PRP preparation with different compositions: a low platelet ($382 \times 10^3/mm^3$) and low leukocyte ($0.6 \times 10^3/mm^3$) process with one spin step at 1,500 rpm for 5 minutes (10 ml whole blood); a high platelet ($940 \times 10^3/mm^3$) and high leukocyte ($17 \times 10^3/mm^3$) process with one spin step at 3,200 rpm for 15 minutes (27 ml whole blood); and a double-spin process (1,500 rpm for 5 minutes and 6,300 rpm for 20 minutes) that produced a higher platelet concentration ($472 \times 10^3/mm^3$) and lower leukocyte ($1.5 \times 10^3/mm^3$). Anitua et al. [104] used only one centrifugation spin step and collected the volume immediately above the erythrocyte layer. Blood was collected on sterile tubes (4.5 ml) containing 3.8% (w/v) trisodium citrate, then centrifuged at 460*g* for 8 minutes (PRGF System1, B.T.I. Biotechnology Institute, Vitoria-Gasteiz, Spain, bti-biotechnologyinstitute.com). This protocol obtained a platelet concentration factor of 2.67 above the baseline value. Araki et al. [105] concluded that optimized protocol for PRP preparation was a centrifugation of whole blood at 230-270*g* for 10 minutes, which also contained a low number of leukocytes (4.1%-5.8% of whole blood). Leukocytes appeared to be precipitated when the centrifugation force was $\geq 840g$. In their protocol, whole blood (40-72 ml) was drawn by venipuncture, collected, and divided into 7.5 ml aliquots in 15 ml conical tubes (BD biosciences) and centrifuged. Tubes were centrifuged at 20°C in a refrigerated centrifuge (Kubota 5900; Kubota Co., Markham, Canada, www.kubota-global.net). For the second spin step, an acceleration of 2,300*g* for 10 minutes was applied. The platelet concentration factor was 7.4 times greater than the baseline after removing approximately 1/10 of the PPP and adding EDTA as an anticoagulant. In a recent study by Kececi et al. [106] the first spin (soft spin) protocol was chosen as 250-270*g* for 10 minutes. At the second spin, centrifugation force varied between 300*g*, 500*g*, 750*g*, 1,000*g*, 1,500*g* and 2,000*g* for 10 minutes. The platelet concentration factor increased proportionally to the increase of centrifugal force. The authors opined that a definite platelet concentration might be possible to obtain by adjusting centrifugation force individually according to the personal baseline value.

As reported above, there is a wide variation in the protocols for standardization and preparation of PRP. The centrifugation time and speed affect the number and concentration of platelets and other cell types within the PRP and thus the availability of growth factors, chemokines, pro-inflammatory and anti-inflammatory mediators. There is also a wide biological (between patients) and temporal (day to day) variation

[107]. So, it is difficult to assess which kit for PRP preparation is better and which is worse [108]. Different PRP products might be more or less appropriate to treat different types of tissues and pathologies. The clinical efficacy of PRP remains under debate, and a standardized protocol has not yet been established [109]. So, physicians should select proper PRP preparations after considering their biomolecular characteristics and patient indications [110].

Clinical Applications of Tissue Engineering in Regenerative Plastic Surgery

An expansive toolbox of biomaterial and cell-based technologies stands ready to contribute to the production of tissue engineering solutions to meet clinical needs. However, immense complexity can be found in the various targeted tissues and organs for replacement. Moreover, the injury or disease driving the need for tissue repair or replacement can add levels of complexity. A common challenge encountered in the development of tissue engineering technologies is the need to repair tissue defects or to regenerate organs that have intricate 3D structures. Furthermore, it is challenging to integrate the regenerating tissue with surrounding tissues and to maintain cell viability in large constructs. New important perspectives in the field of regenerative surgery promote tissue regeneration and/or tissue transplantation from the use of stem cells, scaffolds and local release of growth factors and pharmacologically active molecules [111]. The plasticity and the potential usefulness of human ASCs in tissue regeneration has been highlighted, and their use in regenerative surgery is an expanding field [8]. In particular, we reviewed below the current treatments, based also on our experience, and new strategies in the management of soft and hard tissue defects, chronic skin wounds and scars.

Current Treatments in Regenerative Plastic Surgery: The Use of Growth Factors and PRP

The past decade has witnessed a growth in rational management of chronic wounds and some new developments in wound dressings. Various "biological active" therapeutic attempts, mainly the delivery of local growth factors to enhance clinical management of chronic wounds, have been attempted with a limited success so far [112]. In particular, topically applied recombinant human granulocyte macrophage colony stimulating factor and granulocyte colony-stimulating factor (G-CSF) had positive effects on wound healing in small (20 patients), randomized, controlled studies involving venous lower extremity ulcers and diabetic foot ulcers (DFUs) [113]. In addition, becaplermin, a recombinant human PDGF, approved by the U.S. Food and Drug Administration (FDA), was reported to improve healing in patients with advanced stage pressure ulcers and in DFU patients with decreasing incidence of amputations [114]. Several other growth factors that showed multiple functions in wound healing have entered the clinical testing arena. In particular, fibroblast growth factor 2 (FGF-2; or basic FGF, bFGF) was reported to induce granulation tissue formation, epithelialization, and tissue remodeling in addition to positive results in burn and pressure ulcer healing [115]. Furthermore, keratinocyte growth factor-1 (KGF-1; also known as FGF-7), approved by FDA in 2004, was reported to promote healing of skin wounds in mice [116] and effective in the prevention of severe oral mucositis in patients with hematological malignancies receiving high-dose chemotherapy [117]. With the discovery of VEGF more than 30 years ago and with its envisioned potential for vascular therapy, it was presumed to be the solution for therapeutic angiogenesis [118]. Although preclinical testing in different animal models showed its capacity in vascular regeneration, initial clinical trials that used VEGF-A as a proangiogenic factor in various ischemic conditions failed [119]. Local treatment of chronic neuropathic DFUs in humans with recombinant VEGF-A165 (telbermin) revealed positive trends, suggesting biological activity for incidence and time of complete ulcer healing [120], but additional studies will be needed to characterize the safety and efficacy of VEGF-A.

Different efforts have been directed toward the development of dermal substitutes that have qualities similar to native skin [121]. Several companies manufacture "living skin equivalents" of autologous and allogeneic primary cells harvested from explant material. During the last two decades, many of these products have received FDA approval for the treatment of large and diseased skin defects that are refractory to conventional therapy and that have been shown to be effective in a limited number of clinical trials (reviewed in [122]). Actually, in Europe there is a trend to use growth factors, all together, contained in PRP for the treatment of patients affected by lower extremity ulcers and chronic wounds. There are already many publications regarding the use of PRP with/without fat graft in plastic and reconstructive surgery. The authors reported beneficial results from the use of PRP mixed with fat graft in the treatment of chronic lower-extremity ulcers [123–125], loss of substance on the lower limbs [125], and in combination with SVF in post-traumatic lower extremity ulcers [49]. In a study by Crovetti et al. [126], 24 patients with chronic skin ulcers were treated with a series of PRP gel applications. Nine patients demonstrated complete wound healing. There were no adverse effects encountered, and all patients noted decreased pain. Another study by McAleer et al. [127] involved 24 patients with chronic lower extremity ulcers. The wounds were injected with PRP every 2 weeks. Successful wound closure and re-epithelialization were obtained in 20 wounds. These findings were particularly significant because all patients had failed with previous treatments. Kazakos et al. [128] assessed the benefits of autologous PRP gel in the treatment of 59 patients affected by acute limb soft tissue wounds. The study showed that PRP gel treatment was a valuable and effective aid in the management of acute trauma wounds. Powell et al. [129] described the anti-inflammatory properties, with reduced edema and ecchymosis, of the autologous platelet gel in eight women after deep-plane rhytidectomy (face lifting). PRP was also reported to be effective in stopping capillary bleeding in the surgical flaps of a series of 20 patients undergoing various types of cosmetic surgery (face lift, breast size changes, or neck lifts) [83]. The beneficial use of PRP have also been reported in patients affected by Romberg syndrome [130, 131] as well as in bone regeneration of intrabony defects [132, 133]. However, in attempts to regenerate damaged tissues, one has to keep in mind the risks that can be associated with use of factors that promote epithelial migration, angiogenesis and matrix deposition (e.g., recurrence, growth and invasion of a pre-existing tumor).

Table 3. Autologous fat transfer procedure with or without stromal vascular fraction / adipose-derived stem cell enrichment

	Autologous fat transfer	Autologous fat transfer with SVF/ASC enrichment
Definitions	Autologous adipocyte transfer	Multipotent cells isolated from the vascular stromal component of lipoaspirate by enzymatic digestion or mechanical forces
Indications	Soft tissue defects, outcomes of burns, outcomes of scars, breast reconstruction, breast augmentation, volume deficiency due to aging, Parry Romberg syndrome, scleroderma, hemifacial microsomia, wound and lower extremity ulcers, loss of substance.	Enhancement of the fat grafting, wound healing and tissue engineering applications. Possibility to use SVF/ASCs alone in wound and lower extremity ulcers, loss of substance. Possibility to use SVF/ASCs in combination with fat graft in soft tissue defects, outcomes of burns, outcomes of scars, breast reconstruction, breast augmentation, volume deficiency in aging, Parry Romberg syndrome, scleroderma, hemifacial microsomia, wound and lower extremity ulcers, loss of substance.
Harvesting	Vacuum-Syringe-Luer-look aspiration with cannula using tumescent solution Luer-look-syringe aspiration from abdomen, thigh, flank or gluteal region.	Vacuum-Syringe-Luer-look aspiration with cannula using tumescent solution from similar donor sites. Luer-look-syringe aspiration.
Procedures	Centrifugation for separation of blood, supernatant and cells. Removal of supernatant and blood (Coleman technique)	Mechanical forces: (a) Filtration of aspirate fat with 120 μm filter, centrifugation of the filtered suspension at 1,200 rpm for 10 minutes, (b) filtration with 120 μm filter of suspension obtained previously; Enzymatic Digestion: Washing of aspirate with PBS to remove blood, saline solution, and local anesthetics Digestion by collagenase to disgregate cells from adipose tissue Centrifugation of the enzymatic digestion to isolate the SVF pellet containing the ASCs Removal of supernatant and resuspension of cell pellet in saline
Grafting	Blunt cannula injection of small aliquots into different depths from different access sites to correct preoperatively marked defect.	For SVF/ASC-enriched fat grafts, mix SVF/ASCs with fat graft and then perform injection technique similar to fat grafting For wound healing, inject the suspension containing SVF/ASCs in multiple depths in and around the wound; For tissue engineering, seed SVF/ASCs onto scaffolds matrix, then implant.
Outcomes	Good results, but long-term resorption may occur with outcome variability (30%-70%).	Enhancement of angiogenesis, volume maintenance, and improvement of survival fat grafts Reports from Europe and Asia demonstrated that SVF/ASCs may promote healing of problematic wound In tissue engineering, early studies highlighted the differentiation capacity of SVF/ASCs within dermal matrix and collagen scaffold for soft and hard tissue defect healing. In other fields, such as gastroenterology, neurology, cardiology and orthopedics, the SVF/ASCs showed the potential for different clinical applications
Complication	Cellulitis, contour irregularity, lumpiness, prolonged ecchymosis, asymmetry from unpredictability of "take".	Complications similar to those for fat grafting; theoretical risk of cell transformation.
Future	Improved processing and grafting techniques for predictable take.	Soft tissue repair, regenerative cell therapy, improved biological scaffolds.

Abbreviations: ASC, adipose-derived stem cell; SVF, stromal vascular fraction.

Current Treatments in Regenerative Plastic Surgery: The Use of SVF/ASCs

The study of stem cell behavior and their application in tissue engineering is becoming one of the most explored potential therapeutic strategies. On the basis of both in vitro experiments and preclinical studies, SVF/ASCs have been used in different clinical plastic surgery applications (Table 1). In particular, autologous ASCs were reported to be effective in the regeneration of widespread traumatic calvarial bone defects [134]. A 7-year-old girl with post-traumatic calvarial defect was treated with autologous cancellous iliac bone combined with autologous ASCs, fibrin glue, and a biodegradable scaffold. Postoperative computed tomography showed new bone formation and almost complete calvarial continuity. The beneficial application of human ASCs was also reported in cranio-maxillofacial hard-tissue defects [135]. In a pilot study of five patients with Crohn's disease, the treatment of external fistulas with autologous ASCs determined their closure [136, 137]. Moreover, ASCs were also used to repair tracheomediastinal fistulas caused by cancer ablation [138]. The therapeutic potential of ASCs was also used for the treatment of chronic ulcers caused by radiation therapy [67]. Twenty patients being treated for the side effects of radiotherapy, with severe symptoms or irreversible functional damage, received autologous ASCs via repeated hypoinvasive computer-assisted injections. The clinical outcome was systematic improvement or remission of symptoms in all patients evaluated. Although the biomolecular mechanism of cellular therapy is unknown, this therapeutic approach may play a pivotal role in the treatment of intractable ulcers, as well as in chronic wounds [47]. In addition, ASCs were reported to

possess in vivo immunosuppressive properties [139], as demonstrated in a clinical trial in which acute graft versus host disease resolved completely in five of six patients, without side effects, after a median follow-up period of 40 months [140].

The injection of free fat together with autologous SVF/ASCs (Table 3), isolated from a portion of liposuction aspirates, represents an alternative strategy to soft tissue augmentation surgery [141, 142], including also breast augmentation or reconstruction [47, 67], increasing the long-term maintenance of fat graft volume [47, 67]. In fact, SVF/ASC enrichment was proven to induce the secretion of different cytokines and growth factors encouraging angiogenesis and fat graft revascularization [143, 144], as well as pericytes and endothelial cells, contained in the SVF, that directly contribute to vessel formation and angiogenesis [145]. However, it remains unclear whether grafted ASCs may increase the risk of de novo cancer development or recurrence in patients treated for breast reconstruction after post cancer surgery. Preliminary follow-up studies seem to support the efficacy and safety of SVF/ASC enrichment [146, 147].

The Combined Use of SVF/ASCs and PRP in Regenerative Plastic Surgery

Recent studies showed that the treatment with platelet-rich plasma and SVF/ASCs increased survival of grafted adipose tissue. In particular, the combined use of PRP and SVF/ASCs in fat grafting was reported to be effective in the treatment of facial rejuvenation [148], scars on the face [149], soft tissue defects and breast reconstruction [47, 67]. Data from literature reported that PRP-enriched fat grafting was effective in the increase of graft survival as well as SVF/ASC-enriched fat grafting [47, 149–151]; the combination between SVF/ASCs and PRP enhanced the beneficial effect on fat survival and volume maintenance [7, 144, 150], but this is a conflicting result [151]. Bae et al. [152] demonstrated that ASC enrichment of cryopreserved adipose tissue, for soft tissue augmentation in nude mice, was more effective in the increase of fat graft survival compared with SVF enrichment and fat graft alone. Moreover, Por et al. [153] reported no effect of PRP-enrichment on fat graft survival in nude mice compared with fat graft alone.

As concerning osteoarticular pathology, Pak et al. [154] reported at present no cure for painful osteoarthritis in stages 2 and 3. For these patients, the intra-articular injection of ASCs contained in the SVF can be an effective treatment option. Moreover, the harvest of adipose tissue and percutaneous joint injections are considered to be minimally invasive procedures and can be readily accepted by patients. These procedures carry relatively low rates of morbidity and side effects. The injected ASCs were reported to be effective in cartilage regeneration but the combined use with PRP enhanced the symptom improvement. Gibbs et al. [155] showed a case report in which four patients with a history of unresolved symptomatic knee osteoarthritis were investigated for the clinical outcome of an exercise rehabilitation program associated with intra-articular injections of autologous StroMed (i.e., SVF concentrate by ultrasonic cavitation of lipoaspirate) and PRP. Age-related macular degeneration patients were also treated with SVF and PRP showing an improvement of retinal functionality [156].

Effects of Growth Factors and Culture Conditions on SVF/ASC Proliferation, Differentiation, and Fat Graft Survival

As reported, ASCs are able to secrete different growth factors with also angiogenic properties, such as HGF, VEGF, IGF-1, G-CSF, TGF-β, and bFGF [157, 158]. In addition, some pro-inflammatory and anti-inflammatory cytokines are also produced, influencing via paracrine signaling the surrounding microenvironment. The expression of these factors can be modulated by different conditions, such as proliferation, differentiation and hypoxia culture conditions. In fact, Pallua et al. [159] showed that during the adipogenic process, IGF, PDGF, and MMP-9 increased, whereas in proliferation medium decreased. In addition, during differentiation, collagen type VI alpha 1, insulin like growth factor binding protein 6, tropomyosin alpha-1 and 2 were upregulated [160]. During low oxygen culture condition, there was a reduction in the expression of some ECM remodeling proteins, such as osteonectin, collagen type 1α1, collagen type 1α2, fibronectin 1 and TGF-β1-induced protein, as well as type 2 cytokines (IL-13, MCP-1 and CD40), all factors involved in fibrogenesis [157]. In addition, 3D culture systems of ASCs up-regulated 4,000 genes related to ECM proteins (e.g., tenascin C, collagen VI alpha3, and fibronectin 1), cell-adhesion and growth factors (e.g., HGF, VEGF, KGF, b-FGF, MMP-2, and MMP-14) compared to monolayer culture [6, 161]. The SVF, consisting of a heterogeneous cell population, has been reported to possess high angiogenic properties and sustain new vessel formation [162, 163]. In addition, it was reported that SVF components showed also an immunomodulatory capacity [143]; in particular, monocytes and macrophages, contained in the SVF, seem to mediate immune response through the expression of various cytokines [143]. A comparative study between SVF and ASC secretome demonstrated that ASCs expressed high levels of pro-inflammatory cytokines, such as IFN-γ and IL-12 and low levels of IL-1β, IL-8, TNF-α compared with SVF expression. In addition, ASCs expressed high levels of anti-inflammatory cytokines such as IL-10 and IL-13 [143]. As concerning growth factor secretion, SVF expressed much more bFGF and G-CSF compared with ASCs that produced instead high levels of VEGF [143]. PRP, a cocktail of different growth factors (see above), has been reported to induce ASC proliferation in vitro [4, 5, 7, 164] and favor chondrogenic and osteogenic differentiation [32, 165], as well as angiogenesis [6, 7] and fat grafting survival [4, 5, 7, 144, 149, 150]. Fat graft survival, after transplantation, is unpredictable with resorption rates that can exceed 75% of the originally grafted material [166]. In fact, after surgical implantation, fat grafts initially survive by nutrient diffusion from plasma. Thus, smaller grafts have better survival rates than larger grafts because the higher surface to volume ratio. Subsequently, neovascularization, which often occurs early after 48 hours post-transplantation, will begin supplying nutrients to the fat grafts [167]. Large grafts may exhibit higher liquefaction, necrosis, and cyst formation, especially in the central part, due to poorer nutrient diffusion from the plasma and inadequate neovascularization to the central part. The most superficial zone, which is less than 300 μm thick, is the surviving zone. In the latter, both adipocytes and ASCs survive. The second zone is the regenerating zone, in which adipocytes die on day 1, whereas ASCs survive, proliferate and replace the dead ones. The most central zone is the necrotic zone, where both

adipocytes and ASCs die, no regeneration is expected, and the dead space will be absorbed or filled with scar tissue [167]. It has been reported that PRP may increase fat graft survival by (1) providing nutrient support from its plasma components; (2) increasing angiogenesis from multiple angiogenic growth factors, such as PDGF, platelet-activating factor, and VEGF; and (3) enhancing the proliferation and adipogenic differentiation of ASCs in the regeneration zone [167]. Many preclinical studies confirmed an increased angiogenesis after PRP-enriched fat grafting [144, 168]. The adding of PRP in ASC cultures increased the growth factor release by ASCs themselves [165]. Moreover, as reported above, the transfer of autologous SVF/ASCs combined with free fat has become an alternative strategy to soft tissue augmentation, increasing fat graft survival [47, 67]. SVF/ASC enrichment was proven to induce the secretion of different cytokines and growth factors encouraging angiogenesis and fat graft revascularization [143], as well as pericytes and endothelial cells, contained in the SVF, that directly contribute to vessel formation and angiogenesis [144]. Additionally, the secretion of ASC growth factors, such as VEGF, HGF, and TGF-β1, directly contributed to angiogenesis through a paracrine effect [169].

The Use Of SVF/ASCs and PRP: Current Regulation

Strict rules must be applied to biopharmaceutical drugs to ensure high quality standards. Accordingly, the regulations are linked to the related laws, which describe a complex pathway for authorization. Reference is made to the Regulation number 1394/2007 of the European Parliament for advanced therapies, where the definition of ''bioprocess engineering products'' is given. Here, it is specifically said that this definition excludes those products that contain, or are made exclusively of cells and nonvital human or animal tissues and that do not have pharmacological, immunologic, or metabolic action. Included among the advanced therapy pharmaceutical products are those used for gene and somatic cell therapy (Directive 2001/83/European Parliament, Annex I). Cells and tissues are to be considered products of bioprocess engineering if they undergo "considerable manipulation." The same regulation defines the difference between extensive and minimum manipulation and lists which are considered relevant or not. Manipulations that are not considered as bioprocess engineering are as follows: cutting, grinding, shaping, sterilization, centrifugation, soaking in antibiotic or antimicrobial solutions, sterilization, irradiation, separation, concentration or purification, filtration, lyophilization, freezing, cryopreservation, and nitrification. The definition of medicines for advanced therapy excludes non repetitive preparations carried out under supervision of a physician, running a personal prescription for a product specifically designed for that particular patient, without, of course, violating the relevant rules relating to quality and safety.

Looking Forward

The complexity of many tissues targeted for tissue engineering therapies, coupled with confounding factors associated with the clinical context, adds up to many barriers to product development and translation. Indeed, the field of tissue engineering will continue to focus on repairing complex tissues because clinical defects often involve more than one type of tissue. Characterization and consideration of the contributions of other tissue components that are often overlooked in tissue engineering, such as the lymphatics, will present a unique challenge in the development of strategies for composite tissue repair. Additional challenges include developing a deeper understanding of the role of the health status of the patient and the host response in determining the ultimate outcome of a tissue engineering therapy. Similarly, we will need to better understand the in vivo fate of various components of tissue-engineered products, including transplanted cells and the biomaterial-based scaffold, before widespread translation is possible.

Every day in the literature, a new tissue-engineered construct that combines biomaterials, bioactive factors, and cells is described. However, before translation is possible, the complexity of these technologies must be considered carefully with respect to the regulatory pathway. Tissue engineers should remain mindful that pursuit of a complex solution could eclipse a suitable simple alternative and seek to adopt the simplest approach possible to achieve the desired results in people. Additionally, technologies presenting an insufficient market base or a complex regulatory pathway may not generate sufficient funding to drive them from preclinical development to clinical implementation.

Recently, increasing effort in the field has been invested in the collaborative development of approaches for tissue regeneration with a focus on translation to the clinic as rapidly and as safely as possible, as demonstrated by the Armed Forces Institute of Regenerative Medicine (http://www.afirm.mil/). Some approaches seek to leverage existing clinical products or components thereof to potentially mitigate the regulatory burden for clinical translation. In other cases, staged approaches for tissue repair or regeneration are being developed, which will use the body as a bioreactor to facilitate production of complex, vascularized tissues to fill defects. At the same time, clinical development continues for technologies that are already positively affecting the lives of patients. Indeed, the capability of the field of complex tissue engineering to develop technologies to advance patient care has already been demonstrated through a variety of products and clinical successes. The impact of the field will continue to grow with the collaborative development of tissue-engineered products that present simple solutions to complex problems.

The combination of our current knowledge in basic biology, the identification of the limits of past clinical trials as well as translational research that includes development of improved animal models, harnessing of new technologies for more accurate imaging, and biomarker-based diagnostics will provide a strong basis to advance viable clinical approaches for regenerative medicine. Moreover, larger, randomized, controlled, double-blinded trials are needed to optimize the delivery protocols and to confirm the early observations of promising clinical outcomes.

Author Contributions

P.G.: Conception and design, manuscript writing, data analysis and interpretation, provision of study material or patients,

final approval of manuscript; M.G.S.: Manuscript writing, data analysis and interpretation; A.B.: Collection and/or assembly of data; A.O. and V.C.: Final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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