The Influence of additional bone substitutes on tooth graft healing

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Abstract

Bone regeneration in dentistry is often mandatory in order to provide edentulous patients with implant-supported rehabilitations, in the presence of alveolar bone defects. Bone reconstruction and alveolar ridge preservation procedures are often challenging and the use of grafting biomaterials of various origin has become very popular among clinicians. Autogenous bone graft is still considered the gold standard material in many situations due to its osteogenic, osteoconductive, and osteoinductive properties, but the limited availability and the need for a harvesting site, which increase patient discomfort, represent some disadvantages. In order to address such drawbacks, over the years, many types of bone substitutes (allografts, xenografts, alloplasts, and various combinations) have been used. They all have osteoconductive features but lack osteogenic property, and only allografts were claimed to be slightly osteoinductive, due to the presence of BMPs. In addition, the degradation rate of the various bone substitutes (which in turn determines the rate of graft replacement with newly formed bone) can be highly variable, with bovine-derived xenograft able to persist for many years within the grafted site. Among the latest products on the market, a device called Tooth Transformer® has been developed, which is able to process the teeth extracted from the patient, transforming them into partially decalcified dentin/enamel granules ("tooth graft"), which can be used as a self-derived bone substitute. The advantages of this material are the total biocompatibility (being of autologous origin), the osteoinduction property (due to the presence of BMPs in the tooth graft matrix), and the proportion of collagen and hydroxyapatite very similar to the bone tissue.

Furthermore, the teeth that need to be extracted become a valuable resource instead of being wasted, as often happens.

This system has proved effective, allowing the patient to obtain very good bone regeneration. Furthermore, it is possible to use the tooth graft together with other bone substitutes already present on the market, in the case of large defects that require a large amount of graft volume. In this regard, it is useful to know whether the addition of other bone substitutes alters bone regeneration for better or worse.

To answer this question, it was decided to carry out this project, which involves the histomorphometric analysis of bone samples regenerated with tooth graft alone (obtained with the Tooth Transformer®) and tooth graft combined with other bone substitutes.

Specifically, the histomorphometric analysis will examine the following points:

- Amount of newly formed bone
- Amount of residual tooth graft
- Size of the residual tooth graft granules
- Amount of residual bone substitute
- Size of the granules of the bone substitute
- Presence and quantification of bone-bone substitute spaces
- Quantification of trabecular spaces

The data collected will allow us to assess:

- The bone regeneration process in particular, in which they will be able to give an estimate of the quality of the bone in terms of absolute quantity and compactness, and whether there is a difference depending on the biomaterial used in combination with the tooth graft.
- The structural integrity and mechanical properties, since the presence of spaces between the bone substitute and the newly formed bone can be associated with a lower resistance of the newly formed tissue.

These analyses will be performed on two groups of samples each: one group represented by biopsies of bone re generated using only tooth graft (control) and a second group regenerated with tooth graft in combination with a second bone substitute (test). The most common bone substitute that will be investigated in this study is deproteinized

bovine bone mineral (DBBM). All biopsies will be obtained after a healing period of 4 to 6 months. If possible, results obtained at different healing times will be compared.

The hypothesis is that the addition of DBBM will increase the total volume of bone (newly formed bone+residual DBBM graft), but will decrease the amount of newly formed bone.

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To my Family
and my Love

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1 Introduction

1.1 Thesis statement and starting point

Approximately 2 years ago, I watched a speech by Paul Sharpe, who is the head of the center for craniofacial & regenerative biology at the dentistry department of King's College London. His speech was focused on Bio-tooth which means whole tooth replacement, and I found it very interesting, and it was the starting point of this dissertation. So, I started to connect with their department to do my thesis, but in that period of time there were some limitations regarding COVID.19 and it was not possible for me to change my location for approximately one year. By considering these points, I started to discover more in this area and tried to find a department to collaborate with them. I found the Department of Biomedical, Surgical, and Dental Sciences at the University of Milan. I found Prof. Del Fabro and Prof. Taschieri. They suggested I skip my interest in Bio-tooth since it takes a long time and it seems impossible to do such research in a short period of time. So, we decided to work on bone regeneration in dentistry. In this case, I had the opportunity to get familiar with this area and, most importantly, do practical work in the laboratory. Also, I knew that many bone defects are usually caused by tumor resection, congenital malformation, trauma, fractures, surgery, or periodontitis in dentistry, and it was interesting for me to play my own role and take a small step in an enormous world of regenerative medicine. Finally, we decided to work on applying biomaterials as a graft and evaluate the process of altering bone regeneration.

All in all, these experiences and information convinced me to follow this way. I believe that, in general, regenerative medicine has the potential to impact the whole spectrum of health

care, and what is more important than health and how does it affect our lives? This reality is a hundred times more important when we consider patients with special conditions. Generally speaking, by considering all the challenges in this field, I believe that such investigating in this area will change the quality of life. As a young researcher in this huge world, I hope this thesis will be a small step and solve a problem.

1.2 COVID Pandemic Situation

Each activity in each context can face some obstacles. Some of the obstacles can be more challenging and others less. In my case, I started working on my thesis in October 2020 and it lasted about 10 months, while at the beginning of the process, the world still suffered from COVID.19. During that time, most of the activities and all the academic spaces, such as universities and laboratories, were temporarily closed. Especially because I worked in the histology laboratory at Galeazzi hospital. I cannot neglect the effects of this pandemic on my daily routine life and also the process of my thesis since I was forced to postpone some procedures. Of course, this pandemic seriously affected our lives, but I deeply believe, according to Kobayashi Issa:

A world of grief and pain,
Flowers bloom,

Even then

1.3 Thesis Structure

This dissertation is divided into six chapters. The first chapter, titled "Introduction", presents the starting points and structure of this dissertation. The second chapter, "Literature reviews", states a conceptual framework for this research and the previous study which is done. The third chapter, "Scope of work", discusses the aims and problems that are going to be tackled by this research and also expresses the main question that forms the thesis. The fourth chapter, titled "Methodology and Data Production", which represents the laboratory and analyzing process. The fifth chapter, "Results and Conclusion", presents the result of this study along with answers to the main questions. Moreover, presents some summarizing remarks and identifies directions for further research.

Chapters sixth " Refrences" and seventh "List of figures and tables" provides the refrences plus list of figures and tables which were used for this study.

2 Literature Review

2.1 Introduction to Tissue Engineering

Tissue and organ failure due to disease, trauma and developmental disorders have been a significant economic and health problem. The use of donated tissues and organs is now a clinical procedure to resolve this problem. However, because of the lack of organ donors, the number of patients on the transplant waiting lists and the ever-growing aging population, relying on donated tissues and organs is not a realistic solution. In addition, due to extreme logistical limitations, many organs from donors cannot be paired, transported and safely transplanted to the recipient within the reasonably short time available.

Tissue engineering (TE) has been introduced to fulfill this critical medical requirements. in fact to developing therapies and products for the reconstruction or replacement of damaged tissues and organs we use TE and regenerative medicine (RM) which are multidisciplinary domains by integrating information and technology from a variety of fields, such as biology, engineering, chemistry, pharmacy, medicinal and material science [1].

Generally speaking TE is a rapidly developing multidisciplinary area in which the concepts of biological sciences and bioengineering such as material science, polymer chemistry, cell biology, and tissue transplantation are combined to create new three-dimensional (3D) biological replacements that simulate the nature of human tissue in order to enhance, restore, or maintain their functions.

The preliminary tissue engineering model involves removing

specific cells from patients through a small biopsy, growing them on a 3D biomimetic scaffold under precisely controlled culture conditions, transporting the structure to the anticipated location in the patient's body, and assisting in the forming of new tissue onto the scaffold, which degrades biologically over time [2].

The general scheme of tissue Engineering is represented in Fig. 2-1 [3].

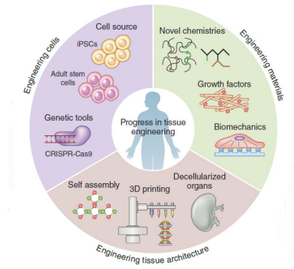


Fig. 2-1 It represents the progression in tissue engineering in terms of cell type growth

2.2 Engineering the architecture of tissue scaffolds

Thanks to two major technologies the ability to create completely controlled 3D architectures for tissue engineering has been significant progress. These technologies are:
1. programmed modular self-assembly
2. 3D bioprinting.
The use of programmed modular self-assembly allows for the quick development of sophisticated synthetic architectures. It's important to know that by u sing DNA strands with sequence complementarity that pair under suitable physical conditions, we can create self-organizing structures. A similar idea has been applied to the macroscale, where biomaterial and tissue building blocks attached with specially engineered programmable DNA glues may be induced to assemble over various length scales ranging from a few hundred micrometers to centimeters. Similarly, degradable DNA glues is compound to single cells to achieve engineered tissue assembly, which can then be degraded using DNase to release the assembled tissue. While nondegradable DNAs allow long-term assembly of biomaterial building blocks into ideal tissue architectures, degradable DNAs are best suited for cases in which living cells can actively fuse into an integral piece after assembly without the need for additional DNA to stabilize the structures [3].

Biofabrication methods such as 3D bioprinting, an extension of existing 3D printing, provide unprecedented flexibility in modifying cells and biomolecules (e.g., proteins and ECMs) with detailed control over composition and spatial distribution to recapitulate the fine form, structure, and design of native tissues [3].

Three-dimensional (3D) printing (or additive manufacturing

(AM)) can create scaffolds customized to the needs of the patient. Bioprinting utilizes additive manufacturing methods to construct 3D structures made up of living cells, biomaterials, and active biomolecules. The homogeneous distribution of cells is one of the key benefits of Bioprinting [4].

Bioprinting is defined as the positioning of biochemicals, biological materials, and living cells for the generation of bioengineered structures of biological and biologically relevant materials with the use of computer-aided transfer and build-up processes. Generally Bioprinting is known as the placement of biochemicals, biological materials, and living cells for the generation of bioengineered structures of biological and biologically related materials by using computer-aided transfer and build-up processes. A general overview of 3D printing processes is shown in Fig. 2-2 [5].

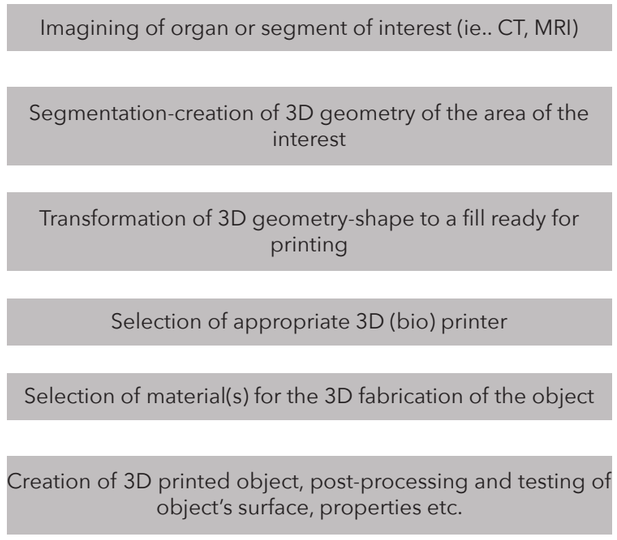


Fig. 2-2 General overview of 3D (bio) printing processes

There are four bioprinting techniques such as Extrusion-Based 3D Cell Printing, Inkjet-Based 3D Cell Printing, Laser-Assisted 3D Cell Printing, and Stereolithography.

2.2.1 Extrusion-Based 3D Cell Printing

In this method to dispense bioinks, extrusion printing implements either an air-force pump or a mechanical screw plunger (Fig. 2-3 a). Extrusion uses a continuous force, allowing for the printing of continuous cylindrical lines rather than a single bioink droplet [7].

This method is the most popular printing method. Also, it's important to know that Ink is extruded into a printhead layer by layer to create a 3D shape. Extrusion bioprinting is operate by Piston, screw, or pneumatic pressure mechanism. In order to print highly viscous bioinks, Micro-nozzle sizes can be used. By use of this technology the control over cell deposition, cell distribution rate, and the speed of the process have significantly improved for scaffold fabrication [4].

2.2.2 Inkjet-Based 3D Cell Printing

The inkjet-based printing technique was initially used for tissue engineering and is somewhat close to traditional 2D inkjet printing [8]. In the ink cartridge, a hydrogel prepolymer solution encapsulating cells (i.e., bioink) is filled. After that, the cartridge is connected to a printing head. The printing head is exerted by a thermal or piezoelectric actuator during the printing process, which could print the bioink while producing controllable droplets in terms of size (Fig. 2-3 b).

Low cost, high printing speed and resolution, and relatively high cell viability (usually 80–90%) are the main advantages of inkjet printing method [9]. However, as the new printing heads are built on microelectromechanical systems (MEMs), thermal or piezoelectric actuation at the opening of the piston produce comparatively minor deformations. Therefore, there is a problem in high-viscosity material that the printing heads cannot squeeze out these materials. In addition, such small deformation does not support high cell density, since high cell density enhance the average viscosity of bioinks and finally leads to clogging of the nozzle. So, the biological materials must also be in a liquid form to permit the formation of low-viscosity droplets [7].

One typical disadvantage is that 3D solid constructs cannot be easily stacked without immediate cross-link processes, so that rapid cross-linkage, such as chemical or ultraviolet mechanisms, are necessary immediately after deposition. To understand this issue, Biomaterials must be chemically altered and reduced viability and functionally characteristics are

needed to do this. Another drawback in inkjet printing called the settling effect was concentrated in recent work [10].

2.2.3 Laser-Assisted 3D Cell Printing

Laser printing is a promising approach for tissue engineering which is developed by Laser induced forward transfer (LIFT) technology (Fig. 2-3 c)[11]. It is necessary to mention that Laser induced forward transfer (LIFT) is a revolutionary alternative to the existing method such as ink jet printing, since it can deposit and position picoliter droplets in a precise way while remaining gentle enough to maintain sensitive structures in the ink [12]. It has significant advantages in the matter of bioprinting and is also called laser-assisted bioprinting (LAB). In general, LIFT assisted printer or LAB has three main components: (1) a pulsating laser source, (2) a donor-slide, to support and propel the printing material, and (3) a receiving substrate to collect and support the printed material. The technology depends on the vaporization of a thin layer of gold/titanium which covers the donor slide caused by the laser. A bubble that propels precursor material on a receiver slide is formed during vaporization. This technique can produce prints in excellent resolution (>20 μm) and cell viability when applying for bioprinting. The precursor is a hydrogel that needs a medium-range viscosity for this process. This technique has been shown to provide precise, multicellular positioning. Receiver Slides can be either natural (biopapers) or synthetic. Also self-assembled cell sheets use for biopapers [4].

2.2.4 Stereolithography

Stereolithography uses UV radiation from the light source (i.e. LASER, Digital Light Processing (DLP)) to cause a photosensitive polymer layer to cross-link.

This method is use for the creation of ceramic composites in polymer matrix (i.e. green ceramics) while it is one of the indirect additive manufacturing (AM) techniques. The advantages of this method generally regarded as the highest resolution and printable freedom technology. Also, beside these benefits, there will also be a drawback. In this case, to allow certain curing depth, the optical characteristics of the powder should match those of the resin. The lateral resolution of the printed piece is necessarily reduced if the powder is reflected in UV light. The handling of high-viscosity slurries used as feedstock is another disadvantage of the stereolithography for ceramics [13]. The schematic of this method is represented in (Fig. 2-3 d) [11].

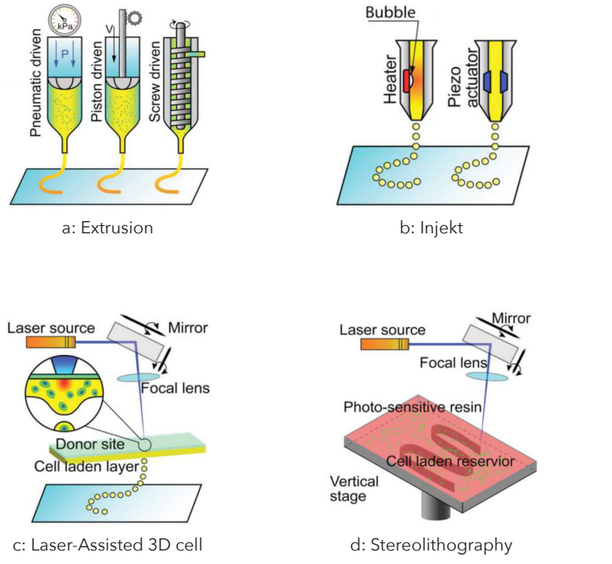


Fig. 2-3 Different 3D printing methods [11]

2.3 In vitro control of tissue development

There is a challenging problem in functional tissue engineering, which is the optimizing in vitro culture environment in order to create three-dimensional (3D) implants that can overcome the in vivo milieu. In particular, in vitro culture conditions can be accurately defined and monitored in order to optimize the structure, composition, and mechanical characteristics of the engineered tissue. In vitro culture conditions include scaffold systems, bioreactors, growth factors, and mechanical conditioning would all have an impact on the development and efficiency of engineered tissues (Fig. 2-4) [14].

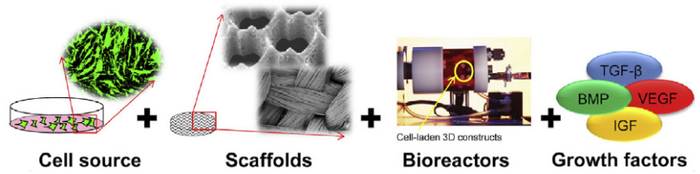


Fig. 2-4 In vetro strategy for engineering functional tissues. Cells, scaffolds, bioreactors, and growth factors used as tool to create functional engineered tissues [14]

Scaffolds are three-dimensional (3D) porous, fibrous or permeable biomaterials considered to permit the following functions:
1. Enhance the interaction of cell-biomaterials, cell adhesion, and extracellular matrix (ECM) deposition.
2. Cell survival, proliferation and differentiation by allowing transport of enough gases, nutrient and regulatory factors.
3. Biodegrade at a controllable rate approximating the tissue regeneration rate in desired culture conditions.
4. Stimulate minimum inflammation or toxicity in vivo [15].

3D scaffolds as tissue models can simulate the structural complexity of living tissues. This point emphasizes the importance of not just the biomaterial used, but also the scaffolds' macro-, micro-, and Nano-architectures. Biomaterials used for 3D scaffolds are categorized as ceramics, glass-ceramics, metals, natural and synthetic polymers, and composites. Biodegradable biomaterials that do not need to be explanted from the organism have recently received considerable attention.

Furthermore, to transport therapeutic agents such as growth factors, proteins, drugs and other substances biomaterial scaffolds are used, and the anchorage of these substances to the scaffold is essential for loading [16]. Bioreactors are considered as in vitro steps of tissue engineering by developing the formation and growth of viable tissues and organs [17].

Bioreactors are laboratory instruments that perform the following tasks:
1. Control cell distribution on 3D scaffolds at the start
2. During in vitro cultivation, efficient mass transferring of gases, nutrients, and growth factors to tissue-engineered constructs.
3. Enable developing constructs to be exposed to convective mixing, perfusion, and/or mechanical, electrical, or other biophysical factors in a controlled way [18].

It's necessary to say that bioreactors are divided into two groups: macrobioreactors and microbioreactors. The first is used mainly to grow functional tissues for implantation, while the second is primarily utilized for testing of drugs, cell response optimization under different stimulations and models of disease [19]. Mechanical conditioning can be defined as

in-vitro application of dynamic mechanical loads (i.e. tension, stress, pressure and/or shear) to cells, tissues or 3D engineered tissue by means of specially designed systems. These basic in vitro culture parameters can be adjusted individually or in combination to satisfy the needs of the specific tissue to be engineered [20].

2.3.1 Mechanobiology

It is a biological field which studies the creation and response of cells or tissues to mechanical signals. This field includes how the cells produce mechanical forces to interact with their environment or their neighbors, and also the mass of cellular responses to mechanical signals. Biology, physics, and engineering have the junction in which mechanobiology helps us to better understand both the development of medical applications such as implants, wound regeneration control, and tissue engineering and also biological phenomena such as morphogenesis, tissue regeneration and cancer metastasis.

2.3.1.1 Mechanobiology in tissue engineering; Bone implant design

Artificial implants ca be used for recapitulation of bone and joint activity in dentistry and orthopedics. These implants are also composed of hard metals (e.g. pure titanium or titanium alloys) attached to native bone. After implantation, implant stability is defined by the body's capability, in a process known as osseointegration, in which expanding and maintains natural bone structure around the implant.

Bone implants osseointegration need both formation and resorption of bone. The same mechanical cues for the reconstruction of native bone are also used to stabilize artificial bone implants. During moving, the native bone is exposed to compression cycles that allow bone formation and bone resorption to increase by osteoblasts and osteoclasts respectively.

Various osseointegration techniques have been developed, such as the covering of the metal surface with hydroxyapatite, which is the bone's main chemical ingredient. Direct cyclic implant loading and loading of the whole bone implants have been shown to greatly increase bone formation at the

implant interface meaning that to enhance osseointegration, combination of common pharmacological therapies with mechanical cues can be useful. The metals used in these implants are currently stiffer than the bones they are intended to replace. Furthermore, the implants do not have the same stiffness differences as native bone architecture. These differences lead to different mechanical conditions between bone structures surrounding the implant and those seen in native bones, which is leading to abnormal patterns of bone formation and resorption and weakening of the bone around the implant. To reduce these effects, implants are designed based on comparable architecture and stiffness to native bones. Also, it's necessary to say that decreasing stiffness causes increasing interfacial stress between the bone and implant, which leads to bone resorption. Implants should present the same pattern and magnitude of mechanical signals to the surrounding tissue as native bone in order to make implants more stable over time and increase osseointegration [21].

2.4 In vivo synthesis of tissue and organs

The in vitro mechanism of engineering tissues is well known, but reproducing organ cellular and structural complexity by using techniques in conventional tissue engineering is extremely difficult. Furthermore, innervation and vascularization are necessary for proper physiological outcomes and both of them cannot be repeated ex vivo. To solve the problems of conventional tissue engineering, an approach was developed to engineer organs and tissue in vivo without exogenous input in the form of cells or growth factors. This approach called the "in vivo bioreactor" (IVB)and it is based on the assumption that providing a single abundant biochemical or biophysical signal, through a simple intervention, in a physically defined environment which can dominate biological noise and invoke intrinsic mechanisms of regeneration and repair [22]. The IVB principle is that biological systems are basically noisy, and thus a single, but suitable signal will totally change the signaling landscape. Though signals known to be important in biology are of biological origin (growth factors, proteins, enzymes, RNA, microRNA, virus), the source of signal in the IVB paradigm may be biophysical in nature or can pair to the cellular microenvironment by mechanosensing elements to start a mechanobiology paradigm and is represent from the outside into the cellular environment.

For the IVB paradigm to be operational and viable, the four conditions mentioned below are necessary (Fig. 2-5):
1. Defining an intrinsic source of plurial or multipotent cells or progenitor cells in the body.
2. Creating a special microenvironment with exception of other cell populations.
3. Presenting a single biophysical or soluble cue to solve the stochastic biological noise.
4. Defining a volume for regenerative process [22].

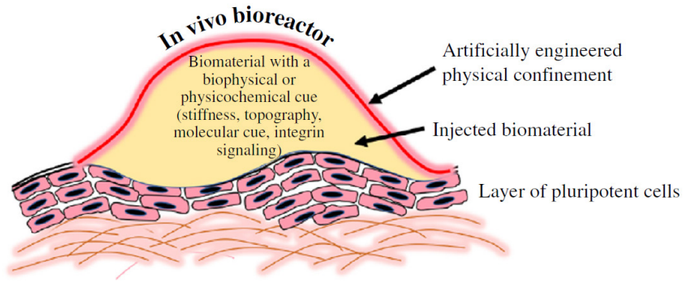


Fig. 2-5 Different main elements of the IVB paradigm and their major roles in ensuring optimum organ or tissue engineering [22]

2.5 Biomaterials in tissue Engineering

The first definition of Biomaterials was develop in the 1980's as "any substance, other than a drug, or a combination of substances, synthetic or natural in origin, which can be used for any period of time, as a whole or as a part of a system, which treats, augments or replaces any tissue, organ or function of the body" [23]. Natural and synthetic biomaterials are a key component of regenerative and tissue engineering techniques. Different types of scaffold are produced from a variety of biomaterials with several manufacturing processes, but the major problem for tissue engineering is choosing suitable materials for the construction of scaffolds. For this purpose, various kinds of biomaterials such as natural or synthetic polymers, ceramics, metals, composites and hydrogels are currently used [24]. There are a range of important factors must be considered to design or determine the suitability of tissue engineering scaffold:

2.5.1 Biocompatibility

The first requirement of scaffold for tissue engineering is that it must be biologically compatible; it must stick to the surface, work normally, and finally migrate through the scaffold,

beginning to proliferate until a new matrix is determined. The scaffold or tissue engineered structure after implantation has to evoke a negligible immune response to avoid a strong inflammatory reaction that can limit healing or cause rejection by the body.

2.5.2 Biodegradability

Tissue engineering allows the body's own cells to replace the tissue engineered construction by passing time. Scaffolds and construction are not considered as permanent implants. Consequently, the scaffold should be biodegradable so that cells can create their extracellular matrix. The by-products should therefore be non-toxic and should be free to leave the body without interference with other organs. A combination of inflammatory reaction and controlled infusion of such cells as macrophages is essential for degradation to occur in conjunction with tissue formation [25].

2.5.3 Mechanical properties

The ideal scaffolds should have mechanical properties which are in accordance with the anatomical site it is to be implanted and should be durable enough from a functional point of view to enable surgical handling during implantation. While this is important for all tissue, it create few problems especially for cardiovascular and orthopedic applications. The first one is about manufacture scaffolds with sufficient mechanical properties for engineering bone or cartilage. The implanted scaffold must have adequate mechanical integrity for these tissues to operate from the time the tissue is implanted to its completion of the remodeling process. The second problem is that healing rates depend on the age; for instance, fractures usually recover in about 6 weeks for a young person with full mechanical integrity not returning about one year after fracture, but slower recovery rates for the elderly. This issue must be included in the construction of orthopedic scaffolds. However, as the area progressed, so much attention could be given to the creation of bone and cartilage scaffolds with similar mechanical properties. Many materials have been developed with great mechanical properties but with high porosity, and many materials with in vitro potential have failed because of inadequate vascularization capability when implanted in vivo. It is clear that a combination between mechanical and porous architectures is enough to enable cell infiltration while vascularization is critical for any successful scaffold [26].

2.5.4 Scaffold architecture

The challenge of core degradation due to lack of vascularization and waste removal from the center of tissue engineered construct is the major challenge in this field. The medium pore size of the scaffold is another main factor. The cells interact mainly with scaffolds on the material surface through chemical groups (ligands). The ligands are normally used in the form of Arg-Asp (RGD) binding sequences in the scaffolds synthesized with the base of natural extracellular matrix (for example collagen), but in the scaffolds with the base of synthetic materials, deliberate incorporation of these ligands is required, for example protein adsorption. The ligand density depends on the surface area, such as the surface of the pores that cells can stick to, while this depends on medium pore size in the scaffold. The pores must therefore be sufficiently large to permit cells to migrate into the structure, which finally links with ligands inside the scaffold, but small sufficiently to create a particular surface which leads to a minimum of ligand densities, so the critical number of cells may effectively bond with the scaffold. So, for any scaffold, an essential number of pores in terms of size are available which depends on the cell type used and the tissue to be engineered [26].

2.5.5 Manufacturing technology

To be clinically and commercially viable, specific scaffolds and tissue engineered constructs must first be economical and then capable of scaling from one at a time in a laboratory to a small group of production. Another important point is defining how a product can be delivered and accessible to clinicians. This would define how the scaffold or tissue engineered construct is stored. Clinicians usually choose off-the-shelf availability without the need for additional surgical procedures to extract cells a few weeks ago from in vitro culture before implantation but for certain tissue types, this is not possible, which will need in vitro engineering prior to implantation [26].

2.6 Biomaterials for tissue engineering and

regenerative medicine

2.6.1 Natural Biomaterials

Natural biomaterials have a wide range of abilities because of their excellent biocompatibility, biodegradability, and remodeling capacity. These features let them to be used in repairing and replacing of injured tissues and organs inside the body. Furthermore, natural biomaterials enhance cell migration, proliferation, differentiation, and adhesion. These properties are critical for tissue engineering because naturally derived biomaterials facilitate cell attachment and migration from the surrounding environment, thus promoting tissue regeneration but these natural biomaterials can also present problems [27;28]. A limitation of natural biomaterials is the immunogenic reaction that can occur after implantation. Another disadvantage, especially for natural polymers, is their decomposing at temperatures lower than their melting point. Natural biomaterials lack the flexibility to create a wide range of shapes and sizes and this limits their use in implants [31]. Several natural biomaterials will be discussed in the following section.

2.6.1.1 Collagen

Collagen is the most common natural polymer present in the body and it is a suitable material for tissue regeneration due to its mechanical properties, biocompatibility, and compatibility with other polymers. Collagen's ability to conduct electricity makes it suitable for a variety of tissue engineering applications. The higher the conductivity of a polymer, the greater cell attachment and proliferation on the scaffold [29]. Collagen's conductivity can be increased by mixing it with other polymers to enhance its tissue engineering functionality [30]. Collagen is created by fibroblast cells, which are gained from pluripotent adventitial cells. The most popular collagen sources for biomedical applications are bovine skin and tendons, porcine skin, and rat tail [32]. Collagen derived from decellularized ECM is also used as a scaffolding material in tissue engineering, and Acellular ECM derived from human/porcine dermis, swine intestine, and bladder submucosa is used in biomedical applications [33]. Collagen-based biomaterials are extremely important because they are both compatible and versatile with body tissues, making them suitable for repairing and replacing body tissues such as tendon, heart valves, skin, vascular grafts, dental, and bones [32].

2.6.1.2 Alginate

Alginate is known as a natural polymer that originates from brown seaweed with high water absorbance potential that promotes cell growth while Alginate has the same construction as extracellular matrix (ECM) which supports cell adhesion with chemical modification [34]. Tissue engineering, wound healing and drug delivery are areas which Alginate is usually used [35]. Generally, Alginate is very biocompatible and it has less or no immunogenic response but since Alginate derived from natural sources it gains many impurities from the environment and it's necessary to know that highly purified alginate represent no immunogenic response while only the purified one can be used in the body [36]. In practice, Alginate has been combined with hydroxyapatite to provide a scaffold for bone regeneration while for creating porous microspheres, the combination of Alginate with hydroxyapatite is necessary and a porous scaffold allows greater cell integration and a much more resistant bone structure when materials decay [37]. Because of its gelling ability, alginate is widely used in drug delivery, and hydrogels are simply dissolvable, enabling them to release drugs predictably. Moreover, alginate is used as a neuro-bridge to treat spinal cord injury by enriching the polymer with two growth factors considered to improve spinal cord repair, so the alginate scaffold released these growth factors and help in repairing spinal cord [38].

2.6.1.3 Chitosan

The polysaccharide polymer chitin can be discovered in the skeleton and internal structures of invertebrates, such as the shellfish exoskeleton and Chitosan is the main derivative of it. Chitosan is a nontoxic, naturally abundant, and renewable biomaterial with valuable properties such as biocompatibility and biodegradability. It also has high biological efficiency and degrades into easily absorbed products [36]. Since chitosan has a hydrophilic structure, it carries some advantages like proliferation, cell adhesion and differentiation in various kinds of cell. Also, for use in tissue engineering and regenerative medicine, it can be design in different forms such as hydrogels, sponges, fibers, sheets, films, etc. [39]. Chitosan as a

scaffold material, can be easily transformed into porous scaffolds, films, and beads. Chitosan has a range of applications, including bone tissue, the central nervous system, and articular cartilage. Furthermore, depending on the application, composites are developed to enhance biomaterial properties, such as bone tissue engineering, in which a microporous chitosan-calcium phosphate composite scaffold demonstrated good osteoblast attachment, increasing scaffold strength and keeping biocompatibility or a chitosan-glycosaminoglycan (GAG) composite is also used effectively to repair articular cartilage [40]. Also in terms of wound healing, Chitosan has been considered since it stimulates the homeostasis and improve tissue regeneration.

2.6.1.4 Silk

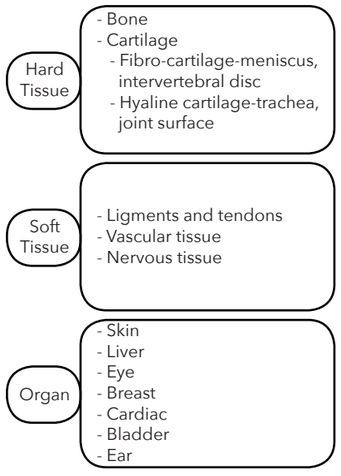


Fig. 2-6 Application of silk based scaffolds in engineering [43]

Silk is a natural protein fiber, and its protein fiber is primarily composed of fibroin, which is formed by some insect larvae to form cocoons. Silkworm silk is used in biomedical applications since it has reliable features such as mechanical properties, biocompatibility and the possibility of manufacturing easily. The elasticity, strength and biocompatibility of silk make it a good candidate for use in tissue engineering and natural fibers can be made into different forms like hydrogels, lyophilized powders, porous scaffolds, native silk mats, and silk microparticles [41]. Silk scaffolds have a wide range of applications in dentistry, including tissue engineering of oral mucosa, dentine, dental pulp, dentine–pulp complex, periodontal ligament/periodontal complex, jaw bone, and TMJ cartilage [42]. In addition, silk-based scaffolds are used in hard tissue engineering, soft tissue engineering, and organ tissue engineering, resulting in different types of tissues (Fig. 2-6) [43].

2.6.1.5 Cellulose

Cellulose is a great biomaterial since it has tunable chemical, physical, and mechanical properties. Since the source material is widely available in nature and easily obtainable, cellulose-based materials are cost-effective for tissue engineering. Biocompatibility, bioactivity, and biomechanics are essential conditions for any biomaterial, and cellulose-based biomaterials meet all of them [44]. In general, cellulose-based materials can be naturally or synthetically derived, and both naturally derived (such as bacterial and plant-based scaffolds) and synthetic materials can be used as biomaterials [45]. In humans, cellulose is not biodegradable. As a result, the regenerated new tissue cannot replace the cellulose. So, one potential disadvantage is that the cellulose occupies space that the tissue cannot while the use of this long-lasting material may have the advantage of providing constant structural support. [45]. Cellulose biomaterials have a variety of uses, including artificial skin and wound dressings, bone tissue, blood vessels, etc. [45]. Furthermore, because of their adjustable surface chemistry and mechanical or physical properties, cellulose scaffolds are an ideal material for 3D nerve cell proliferation and differentiation[46].

2.6.1.6 Fibrin

Fibrin is a fibrillar biopolymer that is produced normally during blood clotting. While the main function of fibrin is hemostasis, it also serves as a provisional matrix during wound healing, and it has properties that make it ideal for use in regenerative medicine; it can transport matrix proteins such as fibronectin and growth factors [47]. Fibrin is very biocompatible since it is a natural polymer in the human body and it defends the body against materials that are not biocompatible [36]. Also, fibrin can use for making cell instructive scaffolds and is often used for stem cell differentiation, stem cell delivery, and angiogenesis induction [48]. Other applications of fibrin include tissue engineering and drug delivery and also as sealants and adhesives in a diverse variety of surgical operations, including neural, vascular, urological, and intestinal procedures [49]. Also, a mixture of fibrin sealants and hydroxyapatite has been used to promote bone regeneration in reconstructive, maxillofacial, and dental surgery; hydroxyapatite is included in these mixtures due to its osteoconductive characteristics [50].

2.6.1.7 Gelatin

Gelatin is a natural polymer produced from the hydrolysis of collagen. These scaffold materials are cheap, easily accessible, and dispersed very well in aqueous solutions [51]. As previously stated, gelatin is easily available and may be derived from a variety of sources, including cow bones, fish, pig skins, and certain insects. Several research on the biocompat-

ibility of gelatin indicated that gelatin, in general, does not induce toxicity, antigenicity, or other unfavorable effects on human cells. Despite that, gelatin has certain drawbacks for biomaterial applications. The major disadvantage of utilizing gelatin is that gelatin-based products have weak mechanical characteristics, lack heat stability, and have a short degradation rate [52]. Gelatin-based materials may not sustain research that needs a longer duration of time, such as cell differentiation, controlled drug release, and wound healing. Furthermore, as compared to collagen, gelatin is very sensitive to a variety of proteases, which may result in fast degradation [53]. These drawbacks, however, can be solved by altering gelatin and creating gelatin-composites to improve mechanical stability, biocompatibility, and bioactivity [52]. Applications in drug delivery system, bioink, transdermal treatment, wound healing and tissue regeneration might be found for gelatin-based hydrogels [54]. Also, Collagen/gelatin hydrogel contains fibronectin doses which are believed to be a promising bio-material for regeneration of the dental pulp [55].

2.6.2 Synthetic Biomaterials

Synthetic biomaterials do not come from natural origins and they are synthesized for desired goals. These biomaterials show advantages for example, they are highly reproducible, they are easily available, and they are tunable in terms of changing different characteristics, such as mechanical properties, rate of degradation, and composition of synthetic biomaterials. On the other hand, they face some disadvantages, like loss of sites for cell adhesion, which limits their regeneration capability in vivo, and the difference between them and natural biomaterials can affect their biocompatibility and ability to promote tissue remodeling. These biomaterials are not natural as said previously thus they can pose the risks, such as immune response and toxicity, but still contain many synthetic biomaterials that are biocompatible and adaptable to the body [56].

Metals, ceramics, nonbiodegradable polymers and biodegradable polymers are examples of synthetic biomaterials. They are used in clinical settings for treatments such as metal hip implants, intraocular plastic lenses, and many more medical applications [28]. The next part will discuss various types

of synthetic biomaterials and their uses in biomedical areas.

2.6.2.1 Polyglycolic Acid

Polyglycolic acid (PGA) was one of the earliest biomedical polymers investigated. PGA has been utilized since the 1970s as a degradable suture. In different applications of tissue engineering, like bone, tendon, cartilage, tooth and backbone regeneration, it is commonly integrated into scaffolds. PGA has disadvantages as it is rapidly degraded and may result in an unwanted inflammatory reaction owing to the resulting glycolic acid rise [57]. PGA was utilized to improve regeneration of the facial nerves. This has been done when the bone marrow stem cells were placed in a PGA tube and observed for the effect of neural regeneration. Since PGA is absorbable, it is suitable for neural regeneration and has FDA approval for nerve grafting [58].

PGA is also used in wound healing and adhesives. Polyglycolic acid sheets, for example, were utilized in dentistry as an open wound healing material for soft tissues and bone surfaces during oral surgery in combination with fibrin glue spray and it's necessary to know that the PGA and fibrin combination produced a considerably stronger sealant than any other biomaterial combination [59].

2.6.2.2 Polylactic Acid

Lactic acid is a naturally occurring organic acid which may be created by chemical synthesis and usually this procedure is related to the hydrolysis of lacronitrile by the help of strong acids [36]. One of the features of PLA is that it is thermoplastic and can be formed into different shapes and can also be used in 3D printing [60]. It is very biocompatible and can absorbed quickly by the body. PLA-based materials have been widely used in orthopaedic and dental applications. For example, in fixation devices such as screws, pins, washers, darts, and arrows in reconstructive surgeries such as those of the mandibular joint; facelifts; thoracic, hand, leg, finger, and toe fractures; ligament reconstruction procedures; soft and hard tissue fixations; alignment of osteochondral and bone fragments; repair of meniscus and hyaline cartilage fixation [61]. These kinds of biomaterials are also used in bioresorbable suture threads, stent coatings and also their usage in

nanomedicine has been considered as a new potential application for the production of nanocarriers for the targeted delivery of hydrophobic drugs [62].

2.6.2.3 Poly Lactic-co-Glycolic Acid

Copolymer of polylactic acid (PLA) and polyglycolic acid (PGA) is known as poly lactic-co-glycolic acid (PLGA). It is one of the common materials for biomedical applications since it can be easily optimized for a variety of uses. PLGA can be changed in terms of form, size, degradation rate, and mechanical characteristics [63]. These biomaterials have advantages like biocompatibility, biodegradability and low toxicity but, their degradation products could create inflammatory and foreign body responses when implanted. In terms of application, it can be used for delivering vaccines, chemotherapeutics, antibiotics, painkillers, siRNA and anti-inflammatory drugs, and also for controlled administration of drugs, proteins, and peptides [57]. Moreover, these types of biomaterials have different applications in dentistry. For example, they can be used for bone fixation, treating periodontal pathogens for better local administration of antibiotics in order to reduce systemic side effects of general antibiotic delivery in the form of PLGA implants, disks, and dental film [64]. It can also be used in buccal mucosa producing and in direct pulp-capping procedures [65].

2.6.2.4 Polycaprolactone

PCL is a polyester that has been used mostly in the tissue engineering area due to its availability, low cost, and adaptability. Its chemical and biological characteristics, physicochemical state, degradability, and mechanical strength are all modified, allowing it to be utilized under severe mechanical, physical, and chemical environments without important loss of features. Because PCL has a relatively long degradation time, it is mostly employed in the replacement of hard tissues in the body, where recovery also takes time. It is also utilized to increase the stiffness of load-bearing tissues in the body [66]. PCL Biomaterials are utilized in drug delivery systems, and there are several types of PCL and PCL-based delivery systems available, including electrospun mats, nanoparticles, microparticles, films, and scaffolds. Biodegradable polymeric films are utilized in dental, vascular, skin, and tendon tissue

engineering applications that require an active surface to act as a replacement for lost tissue or to form a patch for healing. Since pure PCL films are very hydrophobic, they don't have the required properties, but the surface may be changed by creating composites, blends, or copolymers to achieve the desired property [66].

2.6.2.5 Polyetheretherketone

PEEK was originally utilized in the medical area as a fracture fixing material, and it is still used for that purpose today. Despite the technological developments, PEEK remains one of the most popular biomaterials on the market due to its versatility [67]. Its modulus of elasticity is approximately equal to that of cortical bone, making it an excellent option for an interbody device in vertebral fusion applications. Also, PEEK is highly resistant to gamma and electron beam radiation, making its sterilization simple. PEEK is also radiolucent, so the doctor can check that the implant is appropriately positioned for optimum recovery. For the medical applications of PEEK, there are numerous examples, such as dental and cranial reconstruction, tooth replacement, 3D scaffolds, vertebral body replacement, bone reconstruction, and hip implants [36].

2.6.2.6 Polyethylene Glycol

PEG is a linear or branching polyether with a general structure ending with hydroxyl groups that has several properties that make it appropriate for usage in biomedical applications, such as solubility in water and organic solvents, non-toxicity, and hospitable to biological materials [68]. Since it is a non-toxic polymer and creates nonimmunogenicity and nonantigenicity, it can not cause immunogenic responses when brought into the body. PEG is often used in the form of hydrogels which are cross-linked polymer networks that have the capacity to absorb and hold water inside their structure. Because its physical characteristics are comparable to soft tissues, the water content of a hydrogel makes it excellent for tissue engineering applications [36]. In terms of application, PEG is widely used for surface coating of materials which are used for developing artificial implants and the goal is to create a biocompatible interface between the fluids of the body and materials such as ceramics, polymers, and metals, so PEG

is a suitable candidate due to its biocompatibility and water solubility [69]. PEG is also commonly used in drug delivery, cancer diagnosis, wound healing, tissue scaffolding models, cell culture, and tissue regeneration.

2.6.2.7 Polymethyl Methacrylate

PMMA is a lightweight, synthetic polymer which is a cost-effective alternative to polycarbonate when high strength is not required. One advantage is that PMMA does not include potentially hazardous components such as bisphenol-A, which is found in polycarbonate. Furthermore, the synthetic polymer is easier to handle, process, and is less costly than polycarbonate. In practice, PMMA is usually used to repair craniofacial tissue problems such as skin and dentures [70]. PMMA is known by its low toxicity and excellent mechanical properties. PMMA is utilized in a variety of biomaterial applications, including bone cement, lenses, bone replacements, and drug delivery systems. It is also applied to permanently erase wrinkles and scars from skin tissue and such biomaterials are used to replace lost dental roots in dental implants [36].

2.7 Regenerative Dentistry

Tissues like bone marrow, epithelia, bone and connective tissue are continually renewed in the human body. The regeneration ability after injury differs greatly, from tissues with an excellent regeneration capacity like the liver to sensory cells that provide the special senses of hearing and vision that do not regenerate when damaged. In consideration of the response of inflammatory disorders, traumas and malignancies, the desired result is always true regeneration rather than repairing with scarring. How much each tissue can be actually repaired or regenerate is based on the number and type of presented cells, especially stem cells which can differentiate to replace missing tissues. In certain parts of the oral cavity, there is a limited number of cells with a high regenerative potential due to the minimal tissue volume. For example in the dental pulp, decrease in the pulp chamber by age to the volume of tens of microliters [71].

Enamel is a unique tissue since the forming cells known as the ameloblasts are no longer present by the time the tooth is erupting into the oral cavity. This makes dental enamel a unique tissue in that it sits at the interface of hard and soft tissues and depend completely on chemical repair by remineralization from ions in the saliva [71].

When considering the bone, the size and shape of trauma-related defects can be outside the body's ability to repair. These "critical-sized" bone defects cannot be repaired on their own. However, in these conditions, therapeutic intervention may assist the cells in regenerating. In this case, an inductive material and scaffold should be used to enhance the homing of endogenous bone-forming cells and their following differentiation. On the other hand, stem cells can be transplanted into the treatment site. Such therapeutic methods use tissue engineering concepts to restore the function and structure of a given tissue or organ [71].

Regenerative dentistry, which focuses on regeneration of oral and dental tissues, is one branch of regenerative dentistry. It also joins the conceptual triad of tissue engineering, including cells, scaffolds, and bioactive molecules. The cells can come from any number of stem cell sources, including embryonic stem cells, adult stem cells, and induced pluripotent stem cells. The cell type chosen is in parallel with the therapeutic goals. Scaffolds can be designed to move suitable cells, and to deliver signaling molecules to arrange tissue healing. Scaffolds may also be used as a carrier or support for ex vivo cell culturing before the differentiated tissue is surgically transplanted into the defect site. Bioactive molecules like growth factors, genes, and drugs can either be released by the scaffold or delivered separately [71]. It is essential that the scaffold stimulates the features of the target tissue such as biological activity, mechanical integrity, and functionality. To obtain this, the optimum design for regenerative treatments will vary, based on the target tissue. For instance, the requirements of a scaffold for pulpal tissue regeneration are totally different from the one used for alveolar bone augmentation for dental implant placement. The features must meet the regenerative demands as defined from the target site, and then be optimized to achieve the best outcomes [71].

2.7.1 Stem cells used in dental regeneration: classification and properties

Autogenous bone transplantation is now the gold standard procedure for bone defects. It is the preferred method for dental prostheses, periodontal therapy, and dental implants. However, the use of autogenous bone transplantation is significantly restricted due to source restrictions, graft harvest complexity, and donor site morbidity. Furthermore, current treatments for oral diseases can only enhance clinical diagnosis criteria and postpone disease development, but they do not restore lost tissue. As a result, modern innovations are in high demand in order to ensure excellent bone and dental tissue regeneration [72].

Mesenchymal stem cells (MSCs) can be isolated from so many tissues and play an important role in organ development and postnatal repair since they have the capacity to self-renew and differentiate. They also have a potential for differentiation into osteoblasts or odontoblasts and to modulate systematic immunity, and lack of ethical discussion [30]. Both endogenous and exogenous MSCs considered as a big guarantee in regenerative medicine for bone and tooth, among which bone marrow MSCs (BMMSCs) considered more important. In addition, adipose-derived MSCs (ADMSCs) and dental stem cells (DSCs) [72].

2.7.1.1 BMMSCs

Bone marrow mesenchymal stem cells are stem cells with the ability of multidirectional differentiation, can migrate to areas of inflammation and damage while have regulatory effects at these sites to help local recovery and healing. The activity of osteoblasts and osteoclasts can be regulated by BMMSCs using several secreted proteins, transcription factors, miRNAs and other unknown processes [73]. To explain more, maintenance of bone-homoeostasis during adulthood mainly depends on the balance of formation and resorption of the bone. At the cellular level it is strongly balanced BMMSCs by osteoblast differentiation and controlling the activity of the osteoclasts. BMMSC dysfunction was pathologically found to be a key mechanism of cells that is a significant cause of different bone disorders, especially osteoporosis [72].

2.7.1.2 ADMSCs

Adipose-derived mesenchymal stem cells display continuous growth kinetics in in vitro and can be differentiated into numerous categories of cells, including osteocytes, chondrocytes and adipocytes. Also, ADMSCs are often more appropriate than BMMSCs due to the prevalence of lipoaspirates and less morbidity in host within procurement. These are because of their simple accessibility and sufficient availability. Furthermore, unlike BMMSCs, which are susceptible to bone pathological factors, ADMSCs demonstrate functional maintenance in multiple bone pathological conditions, as evidenced by the preservation of cell viability, differentiation capability, and, more significantly, therapeutic efficacy. Recent research has shown that ADMSCs are effective in restoring critical-sized bone defects, optimizing osteopenia, and constructing engineered bone grafts and have a faster proliferation rate than BMMSCs and a greater capacity to retain stem cell characteristics such as self-renewal, proliferation, and differentiation potential, while these cells have been proposed as an excellent alternative to BMMSCs [72; 74].

2.7.1.3 DSCs

Dental stem cells (DSCs) are a valuable source of mesenchymal stem cells for cell therapy and regenerative medicine, even neurodegenerative diseases [75]. DSCs divided in several groups as dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), stem cells from human exfoliated deciduous teeth (SHED), stem cells from the apical papilla (SCAP), dental follicle stem cells (DFSCs), gingival mesenchymal stem cells (GMSC) and human natal dental pulp stem cells (NDPSC) have emerged as attractive cell sources for bone and dental regeneration due to their ease of accessibility and relative abundance [76]. Fig.2-7 represents these types of cells.

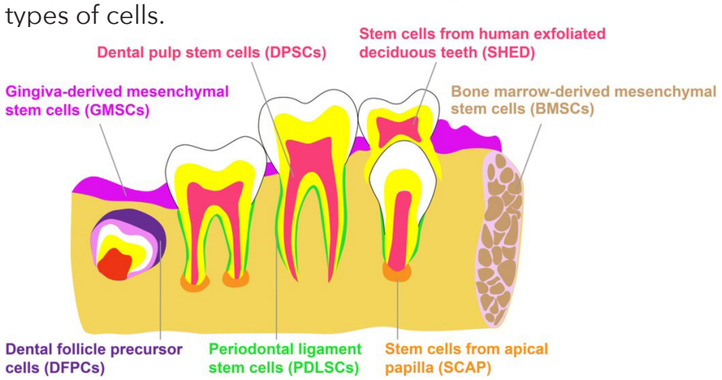


Fig. 2-7 Available human dental mesenchymal stem cells [77]

2.7.1.4 DPSCs

Dental pulp stem cells (DPSCs) are known as a heterogeneous group of cells derived from human permanent third molar pulp. The main difference between them and MSCs is that DPSCs can differentiate into dentin that form odontoblast-like cells. DPSCs have high levels of proliferation, are clonogenic and contain all stem cell properties. They can differentiate between dental and pulp complex tissue and potentially can be used for treatment of pulp tissue in case of injury or infection. DPSCs are multipotent and can be differentiated by microenvironmental factors including growth factors and signaling molecules into adipocyte, odontoblast, neurocyte, etc. DPSCs play a key role in lesions and pulp damage by maintaining the equilibrium between inflammation and tissue repair. DPSCs can differentiate into the components of human teeth, including enamel, pulp tissue, dentin, and cementum. This opens the door for bio-tooth to become a reality [78].

2.7.1.5 PDLSCs

Periodontal ligament stem cells are a source of MSCs from dental tissue capable of regenerating periodontal tissues. They can be differentiated into mesenchymal cell lineages like fibroblasts, osteoblasts, adipocytes, cementoblasts, and certain neuronal precursors [78]. In fact PDL is a type of specialized connective tissue fiber that attaches a tooth to the alveolar bone to support it and PDLSCs are a stem cell subpopulation which was first discovered from PDL. With respect to periodontal defects, PDLSCs are locally transplanted while migrating to PDL and repairing defects with the potential of PDLSCs in periodontal tissue regeneration [72].

2.7.1.6 SHED

A possible non-invasive source of stem cells was provided by SHEDs derived from exfoliated deciduous teeth. SHED shows relatively fast in vitro expansion and distribution. They can differentiate into several cells such as the neural, adipocyte and odontoblast. SHEDs can cause the forming of the bone, create dentin when transplanted in vivo [79]. Like DPSCs, SHEDs are both multidifferentiable and self-renewal, but SHEDs are more proliferated than DPSCs and have been used for dental implant osteointegration alongside hydroxypatite crystal coating because of their bone forming capabilities [78].

2.7.1.7 SCAP

Stem cells from apical papillary (SCAP) can be separated from the adult immature tooth. It means that they are isolated from papilla tissue found in the apical root of teeth. They have a high capacity for migration and proliferation, while producing neural markers without neurogenic stimuli because of specific embryonic origins [80]. Also, SCAPs secrete considerably higher amounts of chemokines, neurotrophins and regenerative growth factors than BMSCs and have immunoregulatory properties [80]. These cells can be implanted in the alveolar socket with PDLSCs which results in forming a bio-root that is able to support a porcelain crown and normal tooth functioning. In addition, these cells are highly proliferative and can be used for different regenerative treatments, especially for the formation of root dentin [78].

2.7.1.8 DFSCs

Dental follicle stem cells (DFSCs) can be isolated from the dental follicle, which is a kind of soft tissue that surrounds the developing tooth germ. They have the ability to form periodontal tissues and have similar properties to that of normal MSCs. DFSCs have the potential to differentiate into periodontal ligament, alveolar bone, osteoblasts and cementoblasts and play an essential role in the developing of tooth [78]. In suitable conditions, DFSCs were shown to have an osteogenic differentiation ability. They have also shown their neural differentiation ability [79].

2.7.1.9 GMSCs

Gingival mesenchymal stem cells are a subpopulation of gingival fibroblasts known as known as immunomodulating sources which are easily available with the ability of differentiating, self-renewal and also anti-inflammatory features [81]. In particular conditions, GMSCs can differentiate into mature osteoblasts, chondrocytes and adipocytes expressing relative cell lineage markers, phenotype and activities. GMSCs often have higher proliferation, which is ideally suited for regenerative medicine and tissue engineering procedures

such as easy to separate while the patients are able to undergo surgical biopsy without concerns about delayed healing [82].

2.7.1.10 NDPSCs

Human natal dental pulp stem cells were reported to have shown abilities for trilineage differentiation, and in comparison to SHED and DPSC, their proliferation activity was higher. The capability of self-renewal and multilineage differentiation potential of SCs are essential in SC therapy. The findings showed that hNDP-SCs can be differentiated into adipogenic, chondrogenic, and osteogenic lineages. Also, hNDP-SCs have also been shown to produce myogenic and neurogenic markers, as well as the ability to differentiate into mature myoblasts and neuroglial cells, demonstrating the complex immunophenotypic characteristics of MSCs [83].

2.8 Tissue and biomaterial sources of bone substitutes grafts used in dental and maxillofacial application

In order to form bone and promote wound healing, bone grafts are known as a filler and scaffold to facilitate this process and make new bones. These grafts are bioresorbable and there is no risk of antigen-antibody reactions. Ridge abnormalities can be caused by surgery, trauma, infection, or congenital anomalies. Bone replacement is suggested to maintain shape, remove dead space, and decrease postoperative infection, finally improving bone and soft tissue recovery. The low quantity of bone is caused by tooth loss, which causes fast degradation of alveolar bone due to a lack of intraosseous stimulation by periodontal ligament (PDL) fibers.

Bone grafting is a surgical technique that replaces missing bone with material from the patient's own body, as well as an artificial, synthetic, or natural substitute [84].

These grafts are suitable candidate since they contain important features like osteogenic, osteoinductive, and/or osteoconductive properties [85].

In this section we discussed about different types of available tissue and biomaterial bone replacement graft options.

Bone substitutes grafts	
Human bone graft tissues	Autografts (cancellous and/or cortical):
	a. Extraoral
	b. Intraoral
	Allografts (cancellous and/or cortical):
	c. Fresh or frozen bone
	d. FDBA
	e. DFDBA
Nonhuman materials	Xenografts:
	a. Bovine hydroxyapatite
	b. Porcine bone
	c. Equine bone
	d. Coralline calcium carbonate
Synthetic materials (alloplasts)	Polymers
	Bioactive glasses
	Calcium phosphates:
	a. Hydroxyapatite
	b. Tricalcium phosphate
	c. Other calcium phosphates (brushite, monetite, CPP)
	Calcium polyphosphate
	Calcium sulfate
	Glass ionomers
	Magnesium-based biodegradable materials

Table 2-1 Bone substitutes grafts

2.8.1 Autograft

Autologous or autogenous bone grafting includes using bone from the same person who is receiving the graft. Non-essential bones, such as the iliac crest, mandibular symphysis, and anterior mandibular ramus, can be harvested for bone. When doing a block transplant, autogenous bone is recommended since there is a reduced chance of graft rejection because the graft is derived from the patient's own body. This form of grafting has the benefit of being osteoinductive, osteogenic, and osteoconductive. The disadvantage of autologous grafts is that an extra surgical site is necessary, which adds to the possibility of postoperative pain and issues [84]. Regarding to the Table 2-1 Autografts used for dental grafting applications may be of extraoral or intraoral origin.

2.8.2 Allografts

Allograft is obtained from humans, and the most common source is a bone bank. The difference is that the allograft is

taken from someone other than the person receiving the graft. It can be collected from living donors or nonliving one. There are three kinds of bone allograft:

- Fresh or fresh-frozen bone
- FDBA (mineralized freeze-dried bone allograft)
- DFDBA (demineralized freeze-dried bone allograft)

The use of allografts for bone regeneration frequently needs sterilization and the deactivation of proteins present in healthy bone. The extracellular matrix of bone tissue is full with bone growth factors, proteins, and other bioactive materials required for osteoinduction and successful bone healing; the desired factors and proteins are extracted from the mineralized tissue using a demineralizing agent such as hydrochloric acid. The mineral composition of the bone degrades, and the osteoinductive agents persist in a demineralized bone matrix (DBM). As a result, it has a negative impact on bone mechanical properties [84]. Fresh and/or frozen cancellous bone shows the maximum level of osteoconductive and osteoinductive potential in comparison with other allografts [86]. FDBA graft tissues are mineralized and used for the treatment of periodontal defects while DFDBA which is demineralized and use alone or in combination with FDBA and autografts [85].

2.8.3 Xenograft

The origin of xenograft bone substitute is from species other than human. Bovine or porcine bone are examples in this case and they can be freeze dried or demineralized and deproteinized. Using xenotransplantation has several biological problems, including an immunological reaction of the host tissue after implantation [87], the possibility of disease transmission (e.g., prions and retroviruses), a shortage of viable cells, and decreased osteoinductive characteristics owing to manufacturing procedures [88].

The most commonly used xenograft in periodontal regeneration is deproteinized bovine bone mineral, also known as Bio-Oss, which is originally bovine bone that has been processed to give natural bone mineral without the organic components [85].

However, Bio-oss is perfectly safe since it is much purified

bone, and allergic responses are quite rare because these types of materials go through a carefully regulated sterilizing process while precise tests are performed to ensure sterility and purity, so they meet high safety requirements.

2.8.4 Alloplast

Alloplasic grafts developed for overcoming the drawbacks of autografts. Since mentioned before the main mineral component of the bone is HA and this kind of graft is made from hydroxyapatite or maybe bioactive glass or marine-derived biomaterials [89]. Among other types of synthetic bone graft, hydroxyapatite is the one used more often because of its osteoconduction, hardness, and acceptability by bone. Currently, the combination of hydroxyapatit with tricalcium phosphate is used, which shows osteoconduction and resorbability [84]. The examples of alloplastic grafts are polymers, calcium phospates (hydroxyapatite, tricalcium phosphates, dicalcium phosphates), bioactive glasses, calcium polyphosphates, glass ionomers, calcium sulphate and magnesium-based biodegradable materials [85].

2.9 Tools and procedures for processing bone substitute from dental origin

First of all it is necessary to know that there are several procedures to make the tooth as a graft material. Below we discuss about some procedures and their drawbacks used by previous researcher:

In a study done by Hussain and colleagues, the bovine teeth were used and the enamel was removed by diamond burs. After that they inserted into defects on rabbit calvaria. The teeth were grind into small pieces by using pestle. Then the pieces were boiled in water for about 2 hours and after that soak in isopropyl alcohol again for 2 hours, then rinsed them with sterile distilled water, finally they dried at 100 celsius degree at the last stage the remnant were smashed by high-speed blender and sterilized by gamma irradiation [90].

This procedure face some problems like we face a long and complex procedures, but it is necessary to eliminate the antigenicity of allogenic tissue and it needs crushing with pestle. So there is a problem of denaturation of proteins due to

mechanical trauma and for sure boiling at high tempreture will cause losing of protein component.

In 2013, Jianan Li and collegues [91] used human demineralized dentin matrix produced by a company. They prepare granules with the size of 10-140μm and mixed with porcine collagen, then frozen and dried anf after that keep in 100 celius degree to form a collagen composite. At last stage they were irradiated with cobalt 60 at 5KGy. So this method is also face drawbacks and it is related to the possibility of it only in industrial level, not in clinical one. Also as ethical point of view the main question in about mixing the tooth (consist of collagen type I) with porcine collagen which means that using heterologous teeth for developing human teeth market. Moreover, there is also the problem of protein denature and losing osteoinductive potential of the tooth due to the treatment at 100 celius degree.

In 2017, Kim and Murata [92] worked on a study related to the use of allogenic teeth. Firstly, the tooth was extracted and chilled with ethyl alcohol, and the pulp and ligament residues were cleaned by using retrograde rotation to make particles with a size of 300-800μm and then the process of demineralizing was done with 0.6M HCL and dehydrated with ethyl alcohol. After The grains are put in distilled water for rinsing and then freeze-dried for 3-5 hours. The important point in this study is the use of allogenic teeth(from the donor) but as mentioned before in the study of Jianan et al [91] , theres is the problem regarding ethical aspect of tooth donation.

Another study, also in 2017, by Minimizato and colleagues [93], worked on 16 patients to evaluate the possibility of creating an autologous demineralized onside dentin particles. In this study, both vital and non-vital teeth were cleaned and soaked in saline buffer solution and then broken up with ice cubes in a high-speed device made up of ceramic blades. Then, the particles with a size of 400-800μm were produced and washed with 1.0M sodium chloride and somewhat demineralized for 10 minutes with 2% nitric acid (PH 1).

In 2010, Murata [94] suggested a method related to the usage of high speed device around 12000 rmp for grinding teeth. It was made up of Zirconium oxide blades with the ability of producing dental particle (0.5 and 5mm). The teeth

kept in freezer at -80 celius degree then cut in pieces, place ice blocks of Saline together, then kept in Nitric acid solution(2%) with PH1 for 20 minutes and finally freeze-dried and filtered. At final stage, the granules were putted into subcutaneous pocket of mice inorder to assess the bone production-after 4 weeks and the overall result was positive.

In 2009, a tooth bank was built in Korea in which the extracted tooth sent there and firstly they processed by the bank, secondly, putted in Ethylene Oxide so it makes them sterile for sure over the time [95].

Although this method is guarantee the qualitative treatment for each patient but contain a limitation about using flammable gas like Ethylene Oxide which is dangerous and require consideration in terms of safety during the procedure.

In general, these methods bring costs, and the material is only available for a limited period after extraction, thus all immediate regeneration procedures are excluded. Furthermore, the use of teeth must be aimed at generating a material with excellent properties compared to other materials on the market, but the particulated tooth which produced by these procedure explained above is treated in the same way as any other grafting material.

2.9.1 Devices on the market

There have been four devices available on the market for using teeth as grafting material. There are Bon Maker, Vacuasonic, Kometabio, and Toot Transformer. We used a tooth transformer in the practical part of this study, and all of the laboratory procedures were done with this device. All of these devices are divided into three steps, which are detailed below;
Step1: Tooth cleaning
Step2: Tooth grinding
Step3: Treatment by devices

2.9.1.1 Tooth cleaning

First of all, the tooth must be cleaned after extraction of any remaining quantity like caries, soft tissues, and restorations (Fig. 2-8). In order to avoid the presence of resins or other

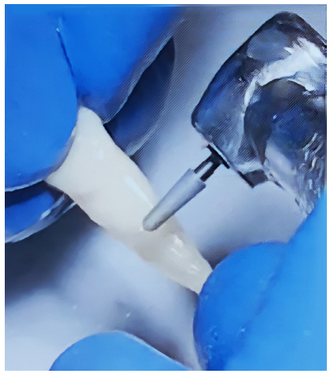


Fig. 2-8 Tooth Cleaning

Fig. 2-9 Sectioning of a tooth with disk

Fig. 2-10 Cleaning of root canal treatment residues using a rotary drill

components in the regeneration material, it is important to eliminate any filling material, even excessive cleaning of the dental tissue on which the reconstruction is positioned. Similarly, the prosthetic and cement are also cleaned. In the Tooth Transformer device, it is required to cut the tooth for grinding (Fig. 2-9). It is advised to clean the root canal treatment residues during the sectioning step, which allows for easier cleaning of small sections that are easily visible under optical magnification (Fig. 2-10).

2.9.1.2 Tooth grinding

The tooth must be crushed in this stage. Tooth transformers employ a multi-use sterilizable system that operates at a low speed that does not allow pulverized dental substances to be lost. Furthermore, the tooth transformer does not enable the insertion of an entire tooth into the grinder, so the sample must be sectioned within dimensions before insertion (Fig. 2-11).

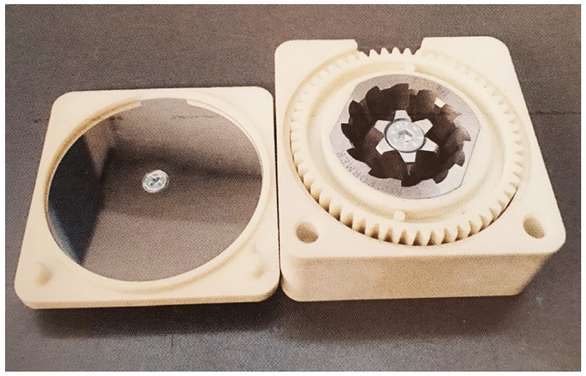


Fig. 2-11 Low speed grinder

2.9.1.3 Treatment by devices

After being inserted into the grinder (Fig. 2-12), the sectioned and cleaned tooth is closed and placed in the device (Fig. 2-13). The disposable part includes a liquid cartridge and a cylinder with a cup for collecting the grains (Fig. 2-14). Both are placed into their corresponding slots in the device, the cartridge is operated by piercing (Fig. 2-15), and the procedure begins once the door is closed and the button is pressed. All of the processes are automated. The granules fall into the collecting basket during the initial step of low-speed grinding. After the automated piercing of the cartridge's lower membrane, the six liquids inside the cartridge tank will fall by gravity and the procedure will begin. Since the proteins denature at around 47 celius degree, the granules are exposed to UVA radiation and ultrasonic vibrations as temperatures that never exceed 43 celius degree. The consumed and polluted liquids remain in the cylindrical container at the end of the operation and may be thrown away. The liquids are made up of 10% hydrogen peroxide, 0.1 M hydrochloric acid, and demineralized water.

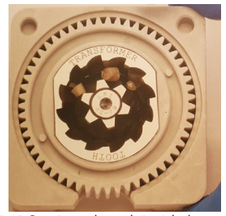


Fig. 2-12 Sectioned tooth inside low speed grinder

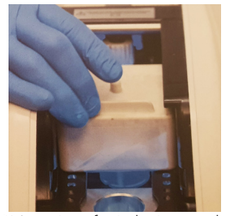


Fig. 2-13 Insertion of grinder into tooth transformer device

Fig. 2-14 Disposable tooth transformer

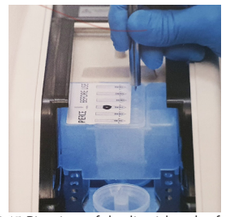


Fig. 2-15 Piercing of the liquid tank of tooth transformer device

3 Scope Of Work

3.1 Problem Statements

As mentioned in the previous chapters, there are various types of bone substitutes for bone regeneration procedures, but they face some problems that we will discuss in this section (Table 3-1).

As an example, in autogenous bone grafts, there is a necessity for additional discomfort due to operative pain at the donor site which leads to loss of blood and also the risk of injury to nearby blood vessels and other tissues, including nerves. In addition, there is a limited amount of available bone tissue, which can be an issue, especially for large defects. Furthermore, the resorption rate of autogenous grafts can be relatively high. In allograft, the drawbacks can be defined as immune rejection, possibility of disease transmission, variable rates of resorption etc. In the case of xenografts, there are problems regarding absence of osteogenicity and osteoinduction, immune response, financial cost, and ethical concerns. Furthermore, the resorption rate is generally very slow, and this can hinder complete substitution of xenografts with autogenous, newly formed bone. In alloplasts, there is a concern regarding the immune response. Moreover, the osteoinductive potential and costs are debatable.

Among the many bone substitutes which are used for bone regeneration procedures and discussed before, xenografts are the most popular, but as mentioned before, xenografts face some disadvantages, while among them, deproteinized bovine bone matrix (DBBM, commercial name Bio-Oss®), is the most frequently used because of its similarity to human bone (Fig. 3-1) This product in human applications shows

	Autograft	Allograft
Advantages	Osteogenic	Osteoinductive
	Osteoinductive	Osteoconductive
	Osteoconductive	No morbidity at donor site
	Biocompatible	High availability
	Sufficient mechanical properties	
	No immune response	
	Xenograft	**Alloplast**
	Osteoinductive	Usually osteoconductive
	Osteoconductive	Low morbidity
	No morbidity at donor site	Enough availability
	Cost effective	No risk of cross infection
	More available	
Disadvantage	**Autograft**	**Allograft**
	Additional surgical phase to obtain autograft	No osteogenicity
	Donor site pain and morbidity	Delayed incorporation
	Limited bone tissue for harvesting	Low availability of healthy grafts
	Increased operative time and cost	Immune response and graft rejection
	High resorption rate	Risk of disease transmission
		Ethical concern
		High cost
	Xenograft	**Alloplast**
	Limit osteogenicity	No osteoinductive nor osteogenic properties
	Delayed incorporation	Risk of immune response
	Availability of healthy grafts	High cost
	Immune response and rejection of the graft	
	Ethical concern	

Table 3-1 Advantages and Disadvantages of bone substitutes grafts

a very slow resorption rate and, therefore, a long-term mechanical support, especially for implant dentistry rehabilitations. Furthermore, there are no known issues regarding biocompatibility and safety, and there is a large availability, too. However, there are some drawbacks related to the product:

Fig. 3-1 Left: Geistlich bio-oss; Right: Human bone
Based on: https://www.geistlich.it/it/dental/sostituti-ossei/bio-oss/vantaggi/

First of all, the financial cost, which increases the overall cost of regeneration procedures for the patient. In addition, Bio-Oss granules, which are used to fill bone defects, often require the use of a covering membrane in order to avoid granule dispersion, which further increases the cost.

Also, we discussed that from a biological point of view, a xenogeneic graft that will not be completely replaced by newly formed autogenous bone, will represent a potential target of future infection, because the vascularization, and consequently the immune defense, will not be as efficient as in autogenous bone tissue. Several case reports (especially patients that underwent maxillary sinus augmentation and ridge augmentation procedures) describe a fast spreading of infection once xenograft becomes exposed and contaminated. Furthermore, xenografts have only osteoconductive properties, and graft maturation requires osteogenic cells and osteoinductive and growth factors to be provided by the host to solve this problem. In the case of Bio-Oss®, which was used in this study, it is important to know that the ultra-porous surface of the Bio-Oss® particles enables interactions that promote bone formation (Fig. 3-2). Many clinicians still prefer to use autogenous bone due to the fact that it possesses all properties required for tissue regeneration, but this often requires a second surgical site for harvesting bone to be used as a graft tissue (one of the major drawbacks of autogenous). Another drawback of autogenous bone is its high resorption rate, much higher than DBBM, which may reduce the long-

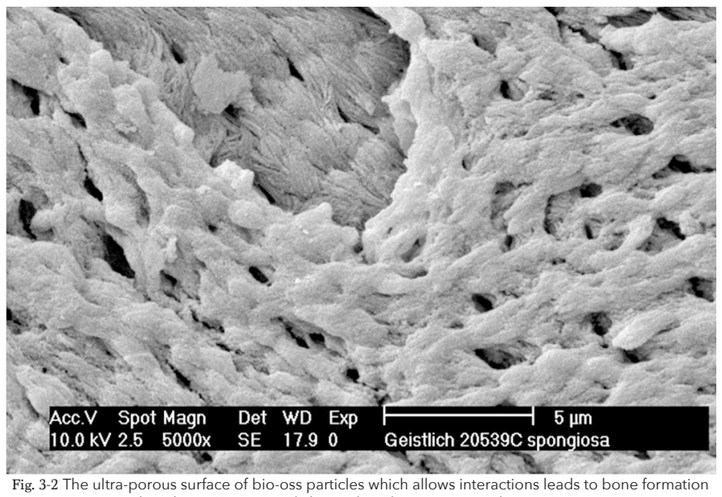


Fig. 3-2 The ultra-porous surface of bio-oss particles which allows interactions leads to bone formation
Based on: https://www.geistlich.it/it/dental/sostituti-ossei/bio-oss/vantaggi/

term support in some cases. So, sometimes DBBM may be the preferred option. In order to minimize the morbidity, DBBM can be associated with a smaller amount of autogenous bone, but this means both a certain degree of morbidity, and an increased financial cost.

Alveolar bone defects are often a consequence of tooth extraction. In fact, alveolar socket healing implies a physiological resorption and shrinkage as a result of bone remodeling after tooth extraction, which is a very common procedure. Alveolar ridge preservation with any type of grafting materials is often performed to limit the dimensional reduction of alveolar bone, before placement of an implant to support prosthetic rehabilitation. One concern of the clinicians, and another reason that led us to do this research, is the wasting of extracted teeth. We wanted to prevent throwing away the teeth. Since the extracted teeth contain small amounts of blood, saliva, or tissue residue, they are considered potentially infectious. In this case, the dentists are forced to throw them away, but it needs a long and complex process which is done by medical waste management companies. They gather teeth and burn them up, but if the teeth contain amalgam, the process is more difficult, because in this case, the teeth must be cleaned in licensed recycling centres dedicated to metal processing. Also, amalgam is made up of mercury, and it can release this hazardous metal into the air, which is a critical issue for the environment and the people who work with these substances. In addition, the relocations made in this process are more costly and time consuming.

Recently, a graft material composed of extracted tooth matrix has been successfully used for bone regeneration procedures. Dentin, in fact, has a composition very similar to human bone, possesses a certain number of osteoinductive factors (bone morphogenetic proteins), and is progressively replaced by newly formed bone, demonstrating a high regeneration potential. In this way, tooth graft appears as a very promising material, because it is totally autogenous, with a very small morbidity, recycles waste tissue (the extracted teeth are not thrashed away), and has a minimal cost, only due to the tooth processing by the dedicated machine which is known as Tooth transformer® in a safe place and prevents additional displacements. So we prevent the invasive process to have availability of autologous graft material.

Moreover, the psychological effect on the patients was considered, since they are aware that no foreign materials are inserted inside their body. Some clinicians, however, believe that, being autogenous, tooth graft might not provide the same support as xenografts, due to a hypothetically higher resorption rate. Therefore, they recommend combining DBBM and autogenous tooth graft for a better result.

The hypothesis of the present study was that comparing grafts composed of autogenous tooth matrix alone and autogenous tooth matrix combined with DBBM (1:1) there are no differences in terms of mechanical support (that is, the total hard tissue % measured histomorphometrically), while it is expected that there would be higher new bone formation with autogenous tooth matrix alone, which would confer a higher protection of the regenerated tissue against future infections as compared with the combined graft.

3.2 Objectives of the study

The main focus of this study is to perform histomorphometric analysis of bone samples from bone defects which have been regenerated with tooth graft alone (processed by Tooth transformer®), versus defects regenerated with the tooth graft in combination with DBBM. This type of analysis and the results are used to evaluate:

- The amount of newly formed bone
- The amount of residual tooth graft
- The amount of residual bone substitute
- The size of the residual tooth graft granules
- The presence of bone-bone substitute spaces and their quantification
- Quantification of trabecular spaces

This data allows us to evaluate features such as bone quality, structural integrity, and mechanical properties.

3.2.1 Evaluate the quality of bone

The regeneration process in bone and the possibility to assess the quality of the bone by calculating the quantity and compactness and whether there is any difference depending on the biomaterial used in combination with tooth graft.

3.2.2 Evaluate the integrity of structures and their mechanical properties

Knowing the presence of any spaces between bone substitutes and newly formed bone may correlate with lower mechanical resistance of newly formed tissue.

4 Methodology and Data Production

In this chapter, we will discuss the practical stage of this study, which leads to obtaining the data. All of these practical parts have been done at Galeazzi Hospital in Milan. This step of the study gives information about how we did the histological process in terms of preparing the samples, staining, microscopic analysis, and finally samples' measurement by a Java-based image processing program. Since we had to prepare the samples, first of all, it is necessary to start with the tissue processing steps.

4.1 Tissue processing

The term 'tissue processing' refers to the steps for transferring an animal or human tissue from fixation to the point that it is totally infiltrated with an appropriate histological wax and ready for section cutting on the microtome. Tissue processing can be accomplished manually or by a tissue processing machine, which is more efficient.

The tissue processing steps for hard tissues and soft tissues are the same. The only difference is that we have one more step for hard tissues (such as teeth and bone) which is known as decalcification. Obviously, we follow the way related to the hard tissue for the purpose of this study. The overall scheme of these steps is represented in (Fig. 4-1).

4.1.1 Fixation

Since the tissues after coming out of the body will decompose for several reasons like lack of oxygen and blood supplies, accumulation of products due to the activity of autolytic enzymes or decay by bacteria, we decided to do the fixation

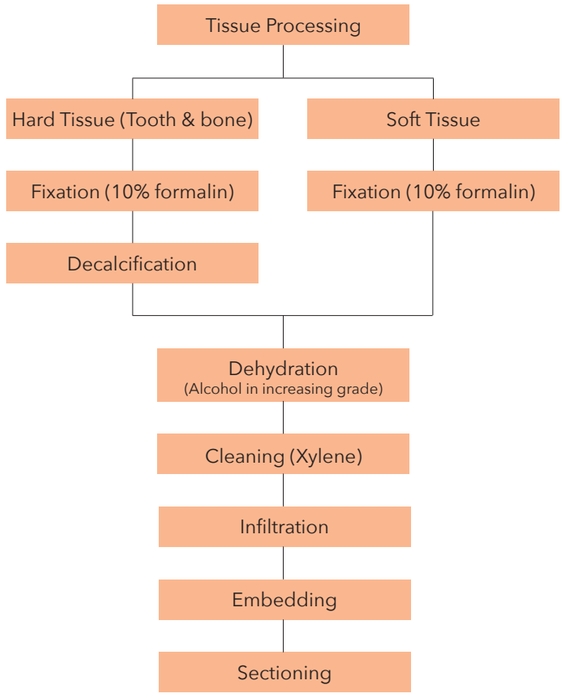


Fig. 4-1 Overal scheme of tissue processing

step to treat tissue which cause hardening and preserve the tissue to protect them. We used Formalin as a fixative by combining 40% Formaldehyde gas with 100% distilled water so, the resulting mixture was completely composed of Formalin. Since we wanted to use 10% of formalin, we combined 10 mL of 100% formalin with 90 mL of purified water. The aim of this process is about making the cross-links between amino acids in proteins to make them insoluble.

4.1.2 Decalcification

As mentioned above, the step of decalcification is for processing the hard tissues which is the materials of this study. The aim of this step is to remove calcium salts from the samples. There are various types of agents and techniques used for decalcification such as: strong mineral acids such as Nitric acid and Hydrochloric acid, organic acids such as Formic and Acetic acid, Picric acid, Citric acid, EDTA and electrolysis. We used EDTA and leave the samples inside decalcifying solution. Since all of our samples were taken from jawbone we

had to wait around 1 week for each group of samples to be decalcified completely. This procedure was carried out under close supervision while we changed the solution day by day (Fig. 4-2).

Fig. 4-2 Decalcification Process

4.1.3 Dehydration

After that, we put all of the samples into alcohol (ethanol) and we increased the concentration of alcohol step by step, as mentioned below. We did that process since molten paraffin wax is hydrophobic and it is necessary to separate water from the samples before we apply wax. As it is clear, ethanol is soluble in water and water inside the samples is replaced by ethanol in each step till we reach the pure samples without water.

The percentage of alcohol concentration and the duration is as below:
- 70% ethanol 15 min
- 90% ethanol 15 min
- 100% ethanol 15 min
- 100% ethanol 15 min
- 100% ethanol 30 min
- 100% ethanol 45 min

4.1.4 Clearing

Although the samples are water free now but it is not possible to put them in paraffin because paraffin and ethanol are completely insoluble. So, we used a solvent which is soluble with both ethanol and paraffin wax. Then, this solvent is replaced with the ethanol inside the tissues and then substitute with paraffin wax. Also, the Xylene agent was used to clear any fat from the tissues to make them suitable for putting in wax just because the fats in tissues can act as a barrier for inserting

samples in paraffin. The duration of putting samples in Xylene is mentioned below:
- Xylene 20 min
- Xylene 20 min
- Xylene 45 min

4.1.5 Wax infiltration

Finally, the tissues were put into wax with a temperature of around 60°C, which is the appropriate temperature for permeating into tissue. There are different kinds of reagents used for this step, but we also used the most common one, which is the paraffin wax-based histological wax. Then, we chilled samples till they reached 20°C to make them harder and prepare them for cutting in a continuous way by microtome. The properties of the wax allow tissues to be cut under the microtome in such narrow strips and keep their elasticity to float in the water when we put them in a warm water bath.

The following is the duration of wax infiltration in our practice:
- Wax 30 min
- Wax 45 min

4.1.6 Embedding or blocking out

Then we molded all of the samples that contained wax into the block. This is necessary because in the next step we have to embed the samples into the microtome for cutting. To do this, we filled the molds with melted wax and the samples were put inside them. Then we needed a cold plate after putting the cassettes on top of the molds to make them harder and solid. Then the blocks, which contain the cassette, removed from the mold.

These sections are then floated in the warm water bath to become flat and then we put them on microscope slides. After drying, they are ready for the next step, which is known as staining.

4.2 Sectioning

We prepared very thin slices like narrow strip or ribbon in

this step from our samples. Since we analyzed our samples with light microscope we made slices around 5-10µm. These sections are then floated in the warm water bath to become flat and then we put them on microscope slides. After drying, they are ready for the next step, which is known as staining (Fig. 4-3).

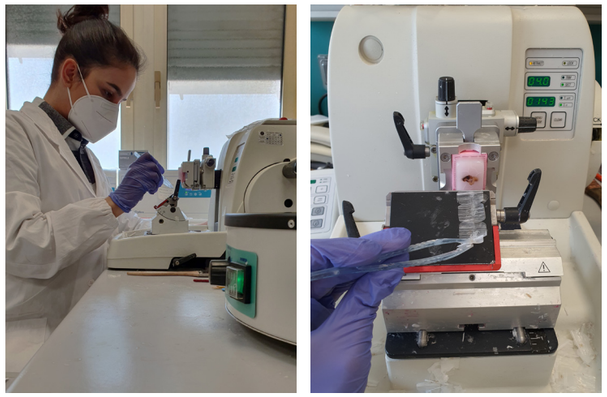


Fig. 4-3 Sectioning Process

4.3 Staining

In the second part of the methodology, before examining specimens under the microscope, we worked on staining approach for our samples since most of the original cells and microorganisms do not have color and contrast under the microscope, and we face a problem determining the cellular structures. So, to solve this problem, we have to do some modification on the specimens to allow us to determine important cellular structures and their characteristics. There are several types of staining techniques but we used H&E or he-matoxylin and eosin.

In this method cell nuclei are stained blue, and cytoplasm or extracellular components are represented pinkish. We accomplished the procedure of H&E staining by the following steps:

- Dewaxing:
First of all, after making paraffin sections, we tried to dissolve all of the wax with Xylene. After preparing paraffin sections, all of the components in terms of cells and tissues are enclosed by paraffin wax, which is hydrophobic. As mentioned before, most of the cells and tissue components do not contain color and are invisible.

- Hydration:
In the next step, we put microscopic slides inside different concentrations of alcohol to separate Xylene, then rinsed them in water. So we obtained hydrated samples, which allow aqueous reagents to penetrate the cells and tissue elements.

- Hematoxylin:
Then applying the Hematoxylin nuclear stain was done. The slides were stained with a nuclear stain, known as Harris Hematoxylin. Hematoxylin has a blue-purple color and stains nucleic acids. In a normal tissue, the nuclei are stained blue, but the cytoplasm and extracellular matrix are pinkish with different shades. It's necessary to mention that we preferred to use regressive staining rather than the progressive one. Just because we obtain better color on our slides. So in this case, we also had to do a differentiation step to remove excess background stains.

- Bluing:
The aim of this step is to change the soluble red component of Hematoxylin into an insoluble blue. So, we again rinsed the slides in water and then made them blue by applying a weak Alkaline solution. After that, we observed that the color of Hematoxylin became dark blue. Then we washed them again and evaluated the quality of the nuclei color and its contrast.

- Differentiation:
As mentioned above, we used regressive staining with Harris Hematoxylin, so a differentiation or destaining step is necessary to remove non-specific background staining and improve contrast. In this case, we used a mild acid alcohol. Then we repeat the bluing and washing process again.

- Eosin:
Then we applied Eosin counterstain to stain the slides with an Eosin aqueous or alcoholic solution. So, many nonnuclear elements are colored in different colors of pink. It means that we can now distinguish between the nuclei and cytoplasm of the cell.

- Rinse, Dehydrate, Clear and Mount (Apply Cover Glass):

After Eosin step, the slide was entered in different concentrations of alcohol respectively to remove all effects of water. Then washed in number of Xylene bath, which makes the tissue transparent and clear. After applying a small coating of polystyrene mountant, a glass coverslip was placed (Fig. 4-4).

Fig. 4-4 Apply cover glass process

In all steps of staining for microscopic slides, we paid attention to creating the right balance of the dyes and preventing overstaining with hematoxylin or eosin, which causes some problems in appearance, like understaining eosin or lighter hematoxylin, respectively. Also, we considered the duration of each step of the process, as mentioned in the Table (4-1):

Xylene	2 Minutes
Xylene	2 Minutes
100% ethanol	2 Minutes
100% ethanol	2 Minutes
95% ethanol	2 Minutes
Water wash	2 Minutes
Hematoxylin	3 Minutes
Water wash	1 Minute
Differentiator (mild acid)	1 Minute
Water wash	1 Minute
Bluing	1 Minute
Water wash	1 Minute
95% ethanol	1 Minute
Eosin	45 Seconds
95% ethanol	1 Minutes
100% ethanol	1 Minute
100% ethanol	1 Minute
Xylene	2 Minutes

Table 4-1 Duration for each step in E&H staining method

Finally, the pictures represented below show the process of H&E staining method that we were done at Galeazzi histology lab, Milan (Fig. 4-5) (Fig. 4-6).

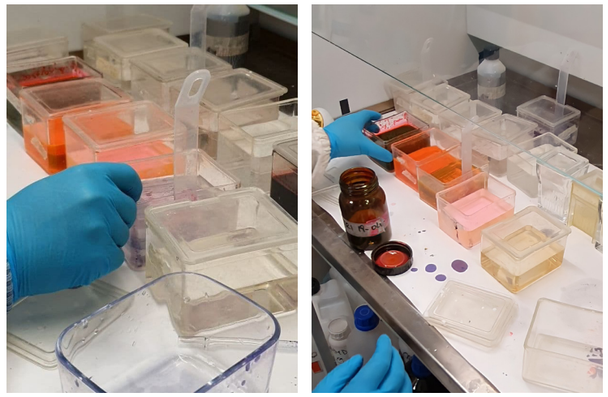


Fig. 4-5 A part of H&E Staining process

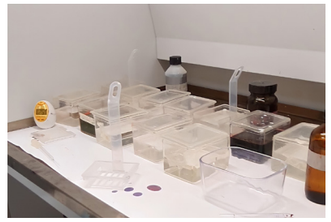


Fig. 4-6 A part of H&E Staining process

4.4 Microscopic Analysis and Measurement

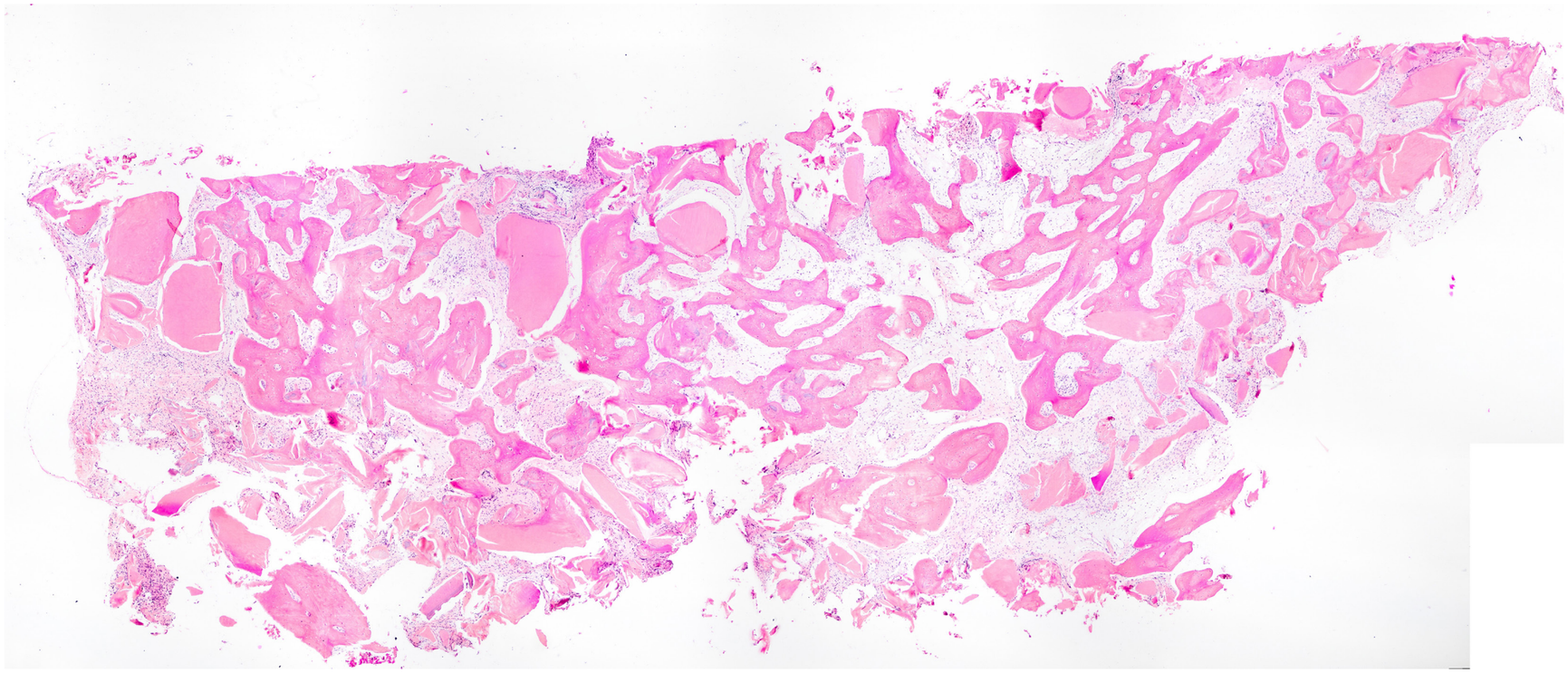
At the next stage of methodology, we analyzed all of the samples under a light microscope and captured the images to make micrographs. Then we put the scale bars on all of the micrographs, which allows us to estimate the size of structures appearing in the images and also calculate the total magnification. So after that, all of the images were ready for analysis by a Java-based image processing program called ImageJ.

As mentioned before, in this study, we worked on 2 groups by dedicating 10 samples to each group. One group, represented by biopsies of bone regenerated using only tooth graft (control), and the second group regenerated with tooth graft in combination with a second bone substitute known as Bio-Oss (test). Moreover, it should be noted that all biopsies were obtained after a healing period of 4-6 months. So, in the first step of analyzing by ImageJ, we tried to separate bone, dentin, and Bio-Oss areas in each sample using ImageJ and measuring their occupied areas. Then we put all the data numeration into excel files.

In the following pages, you will find the pictures and data regarding to the each sample in a table.

4.5 Samples of Control Group

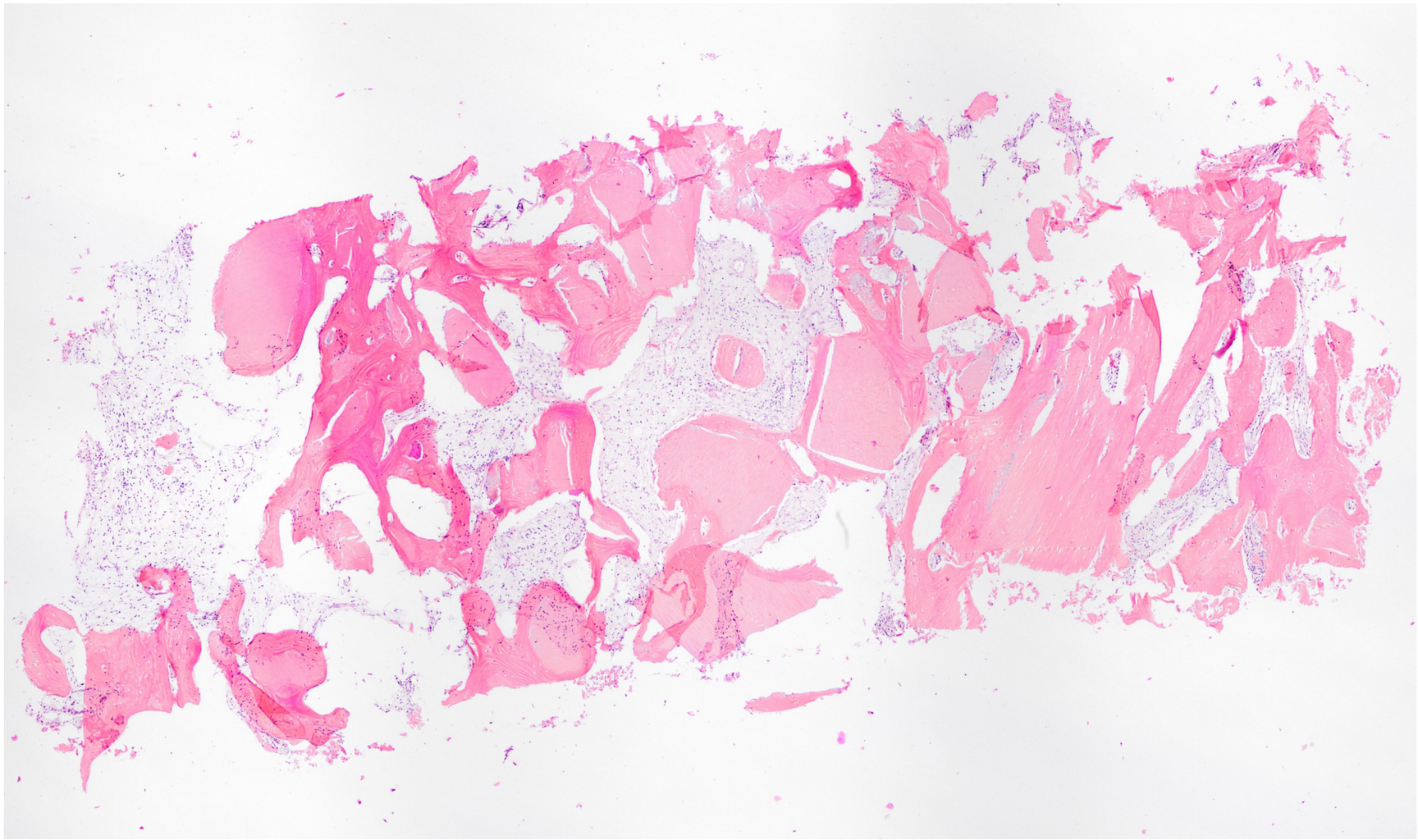
Sample A



Total area	Bone area	Dentin area	Bio oss area	Sup Cal total	% Bone	% Dentin	% Bio oss	% Cal tot	% Partial bone	% Partial dentin	% Partial bio oss	1% Total area
2774.299	820.785	189.769		1010.554	29.585	6.840	0.000	36.426	81.221	18.779	0.000	0.036045141

Fig. 4-7 Sample A with its measurments

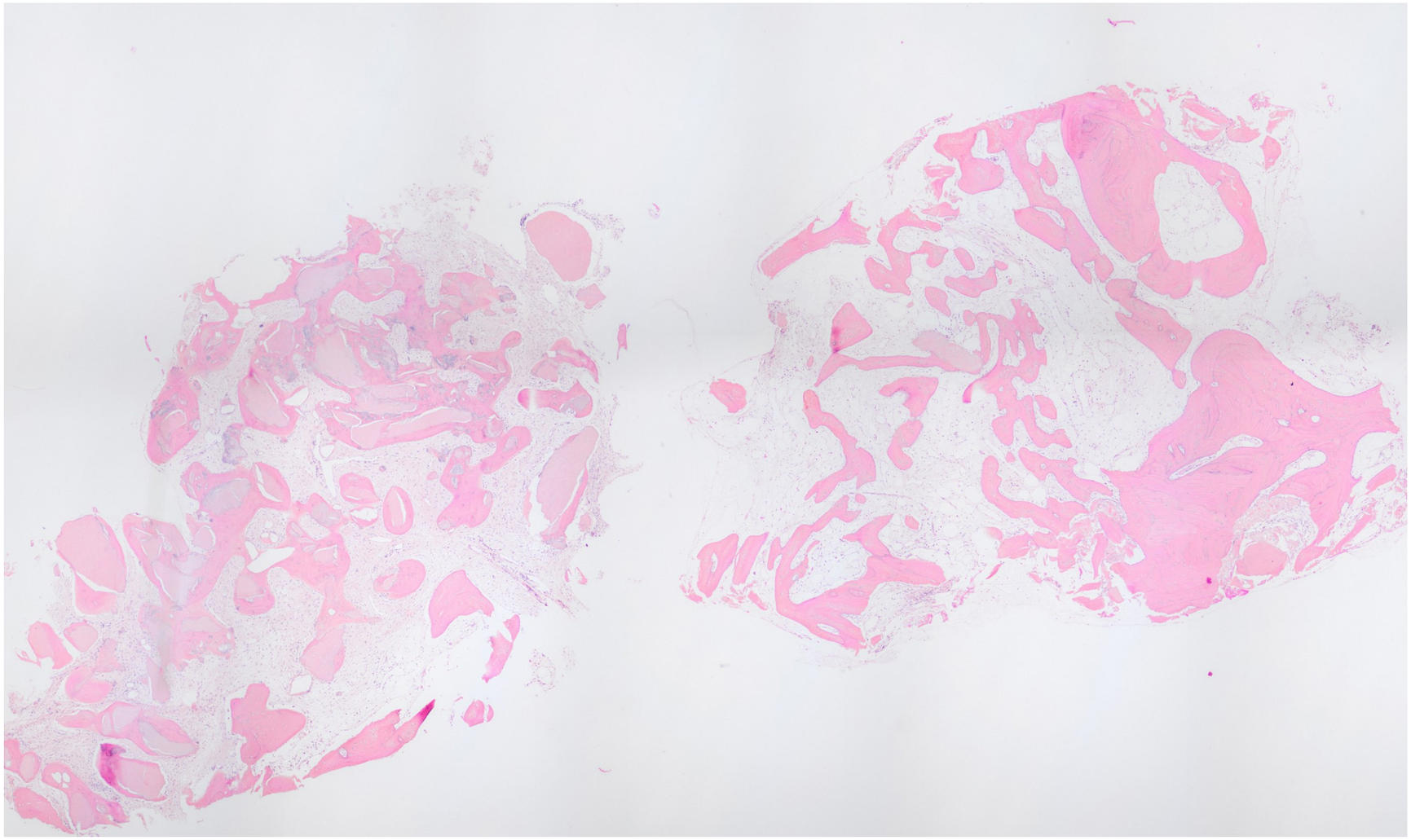
Sample B



Total area	Bone area	Dentin area	Bio oss area	Sup Cal total	% Bone	% Dentin	% Bio oss	% Cal tot	% Partial bone	% Partial dentin	% Partial bio oss	1% Total area
1155.828	439.346	78.869		518.215	38.011	6.824	0.000	44.835	84.781	15.219	0.000	0.086518063

Fig. 4-8 Sample B with its measurments

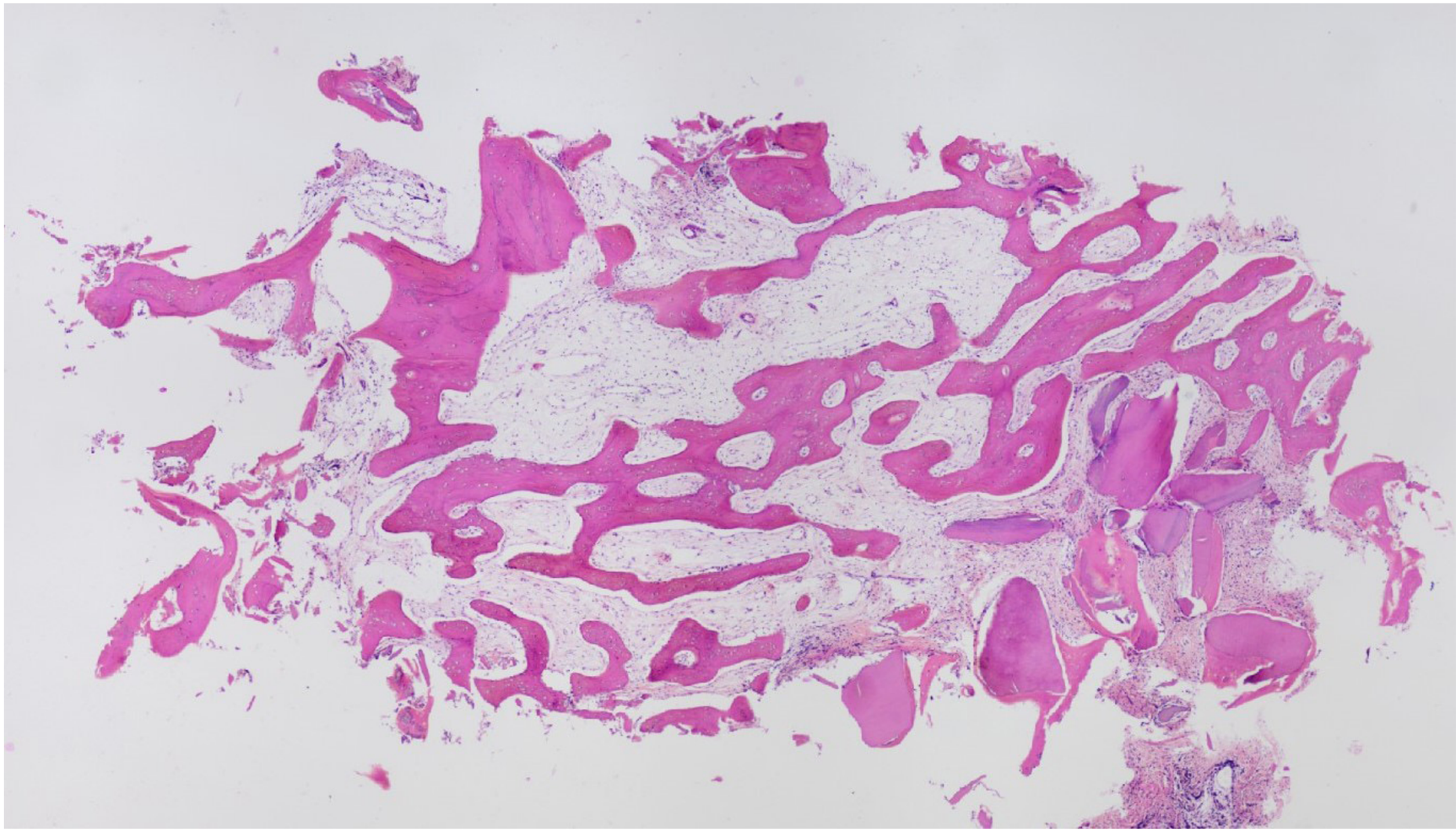
Sample C



Total area	Bone area	Dentin area	Bio oss area	Sup Cal total	% Bone	% Dentin	% Bio oss	% Cal tot	% Partial bone	% Partial dentin	% Partial bio oss	1% Total area
2090.195	635.815	123.237		759.052	30.419	5.896	0.000	36.315	83.764	16.236	0.000	0.047842426

Fig. 4-9 Sample C with its measurments

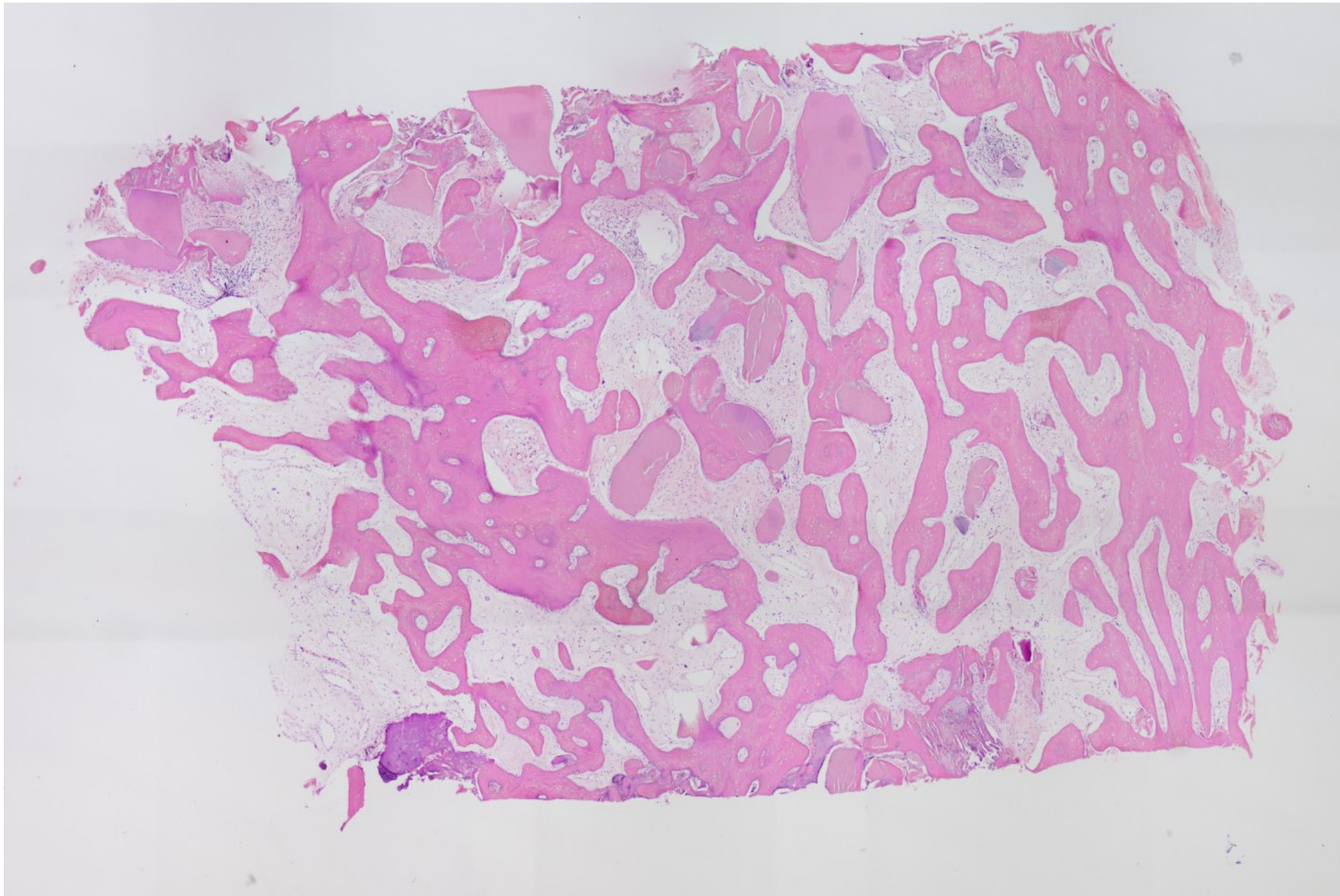
Sample D



Total area	Bone area	Dentin area	Bio oss area	Sup Cal total	% Bone	% Dentin	% Bio oss	% Cal tot	% Partial bone	% Partial dentin	% Partial bio oss	1% Total area
1052.292	353.302	36.066		389.368	33.575	3.427	0.000	37.002	90.737	9.263	0.000	0.095030657

Fig. 4-10 Sample D with its measurments

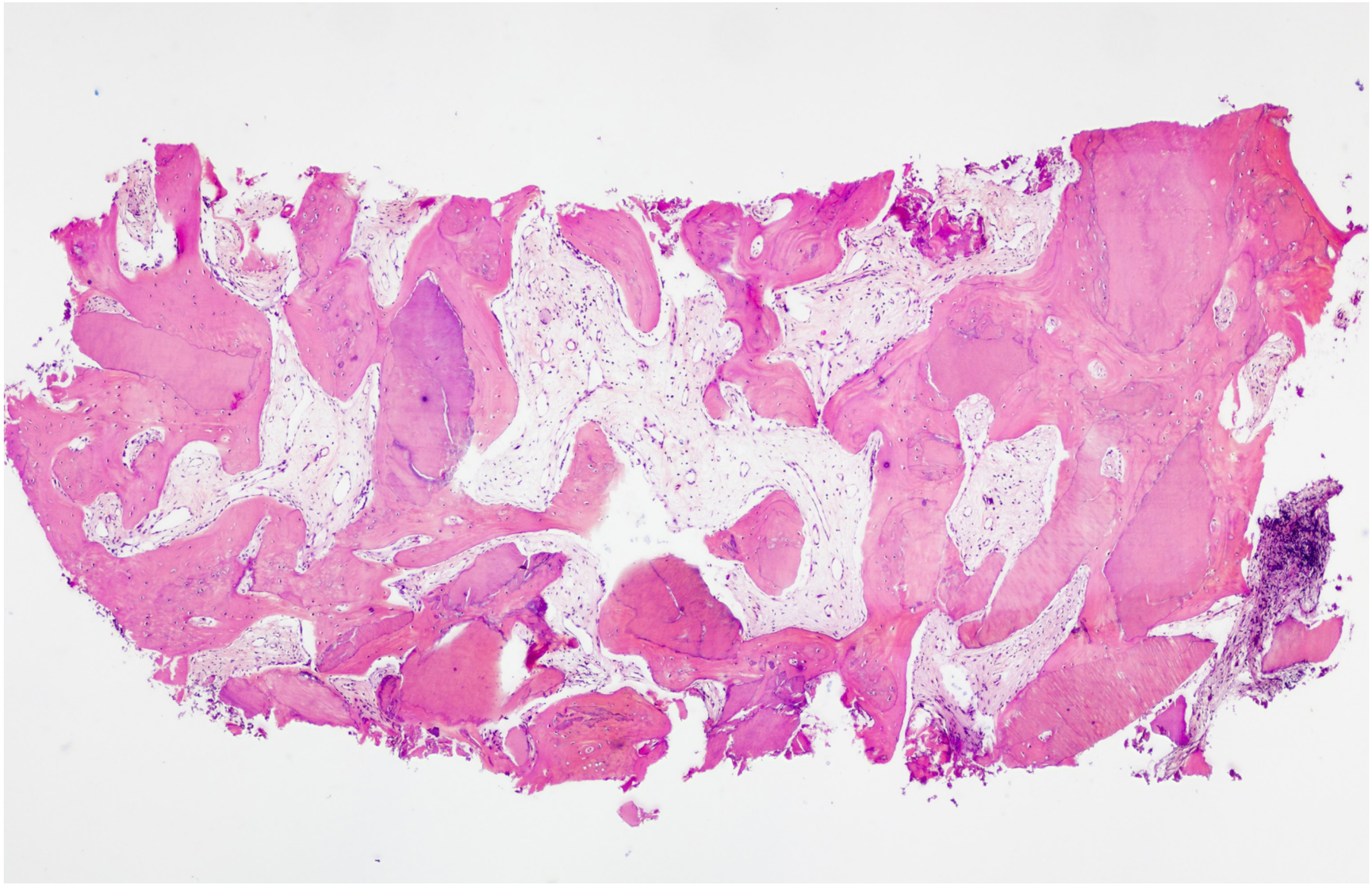
Sample E



Total area	Bone area	Dentin area	Bio oss area	Sup Cal total	% Bone	% Dentin	% Bio oss	% Cal tot	% Partial bone	% Partial dentin	% Partial bio oss	1% Total area
1938.211	816.357	78.736		895.093	42.119	4.062	0.000	46.181	91.204	8.796	0.000	0.05159397

Fig. 4-11 Sample E with its measurments

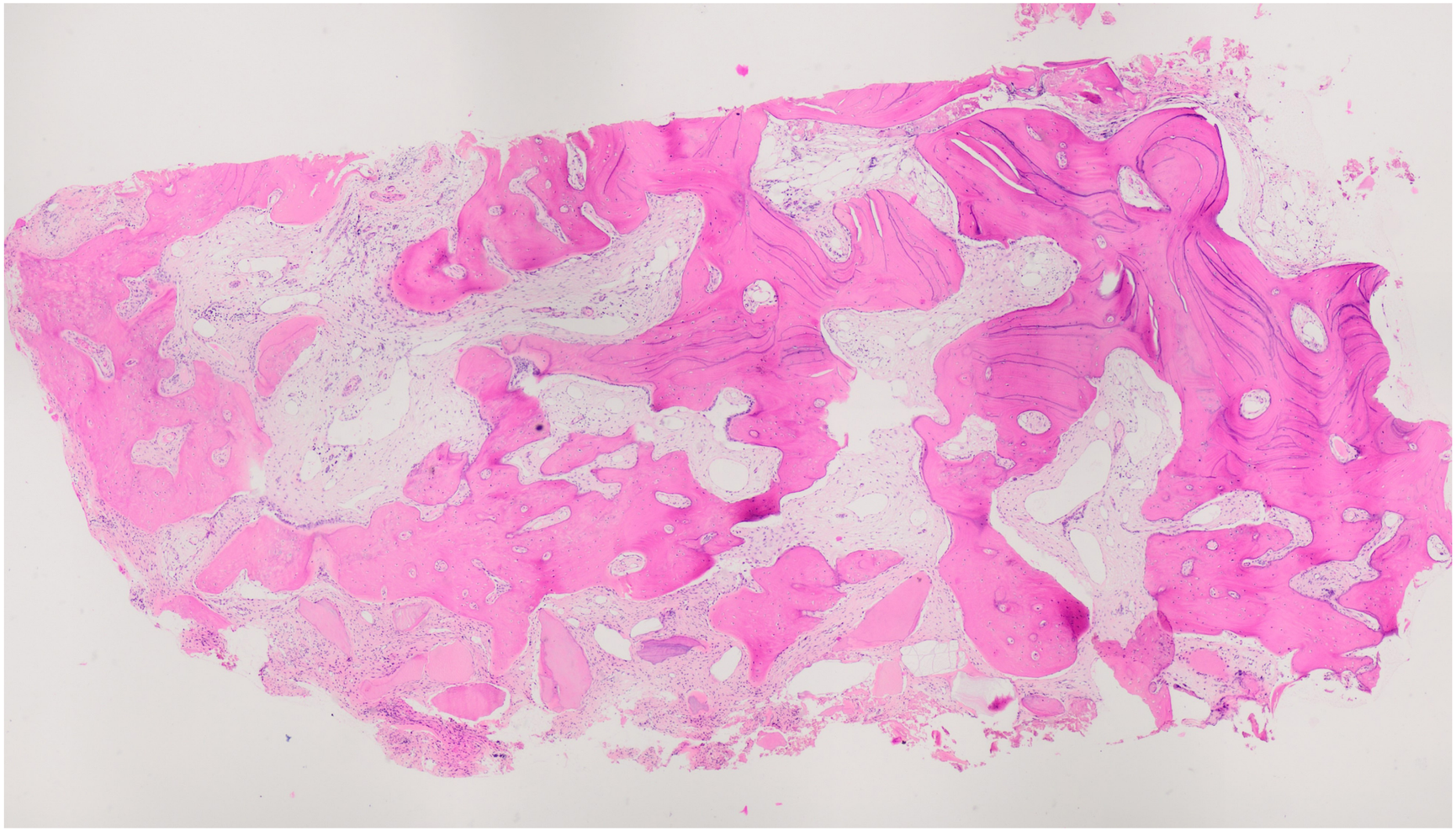
Sample F



Total area	Bone area	Dentin area	Bio oss area	Sup Cal total	% Bone	% Dentin	% Bio oss	% Cal tot	% Partial bone	% Partial dentin	% Partial bio oss	1% Total area
549.94	204.558	90.719		295.277	37.196	16.496	0.000	53.693	69.277	30.723	0.000	0.181838019

Fig. 4-12 Sample F with its measurments

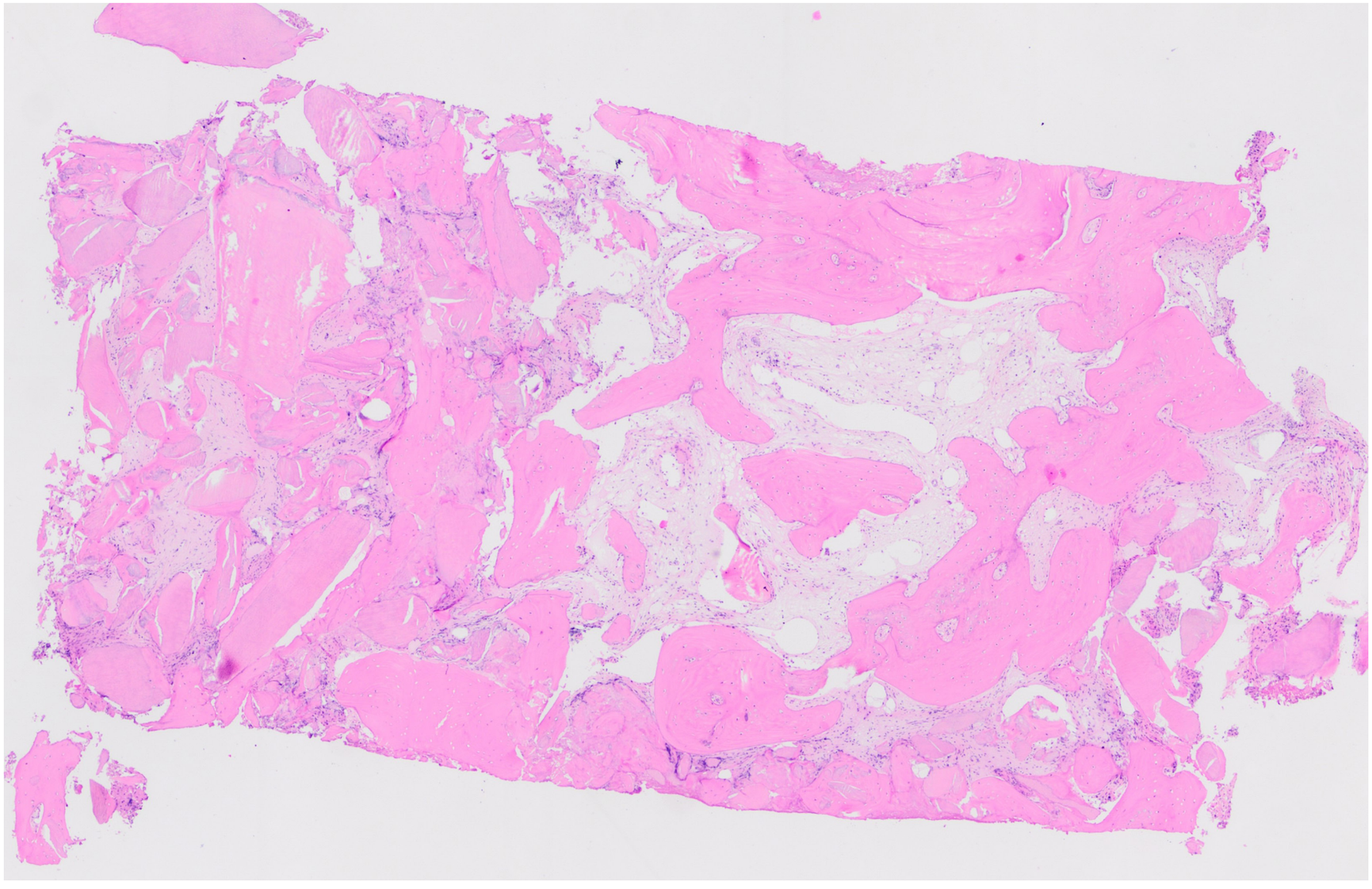
Sample G



Total area	Bone area	Dentin area	Bio oss area	Sup Cal total	% Bone	% Dentin	% Bio oss	% Cal tot	% Partial bone	% Partial dentin	% Partial bio oss	1% Total area
1463.977	692.6	24.744		717.344	47.309	1.690	0.000	49.000	96.551	3.449	0.000	0.068307084

Fig. 4-13 Sample G with its measurments

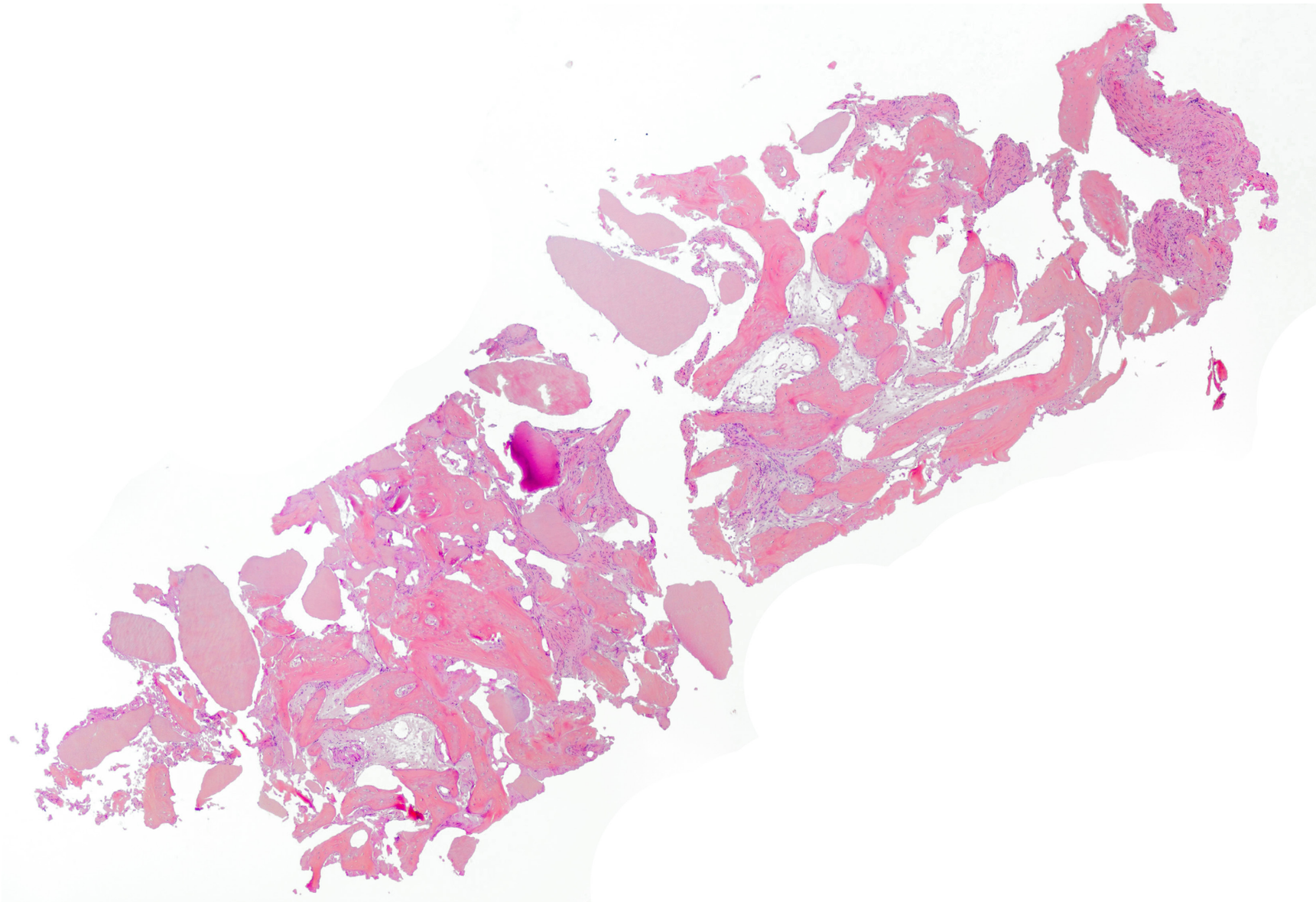
Sample H



Total area	Bone area	Dentin area	Bio oss area	Sup Cal total	% Bone	% Dentin	% Bio oss	% Cal tot	% Partial bone	% Partial dentin	% Partial bio oss	1% Total area
1186.154	411.093	115.451		526.544	34.658	9.733	0.000	44.391	78.074	21.926	0.000	0.084306085

Fig. 4-14 Sample H with its measurments

Sample I

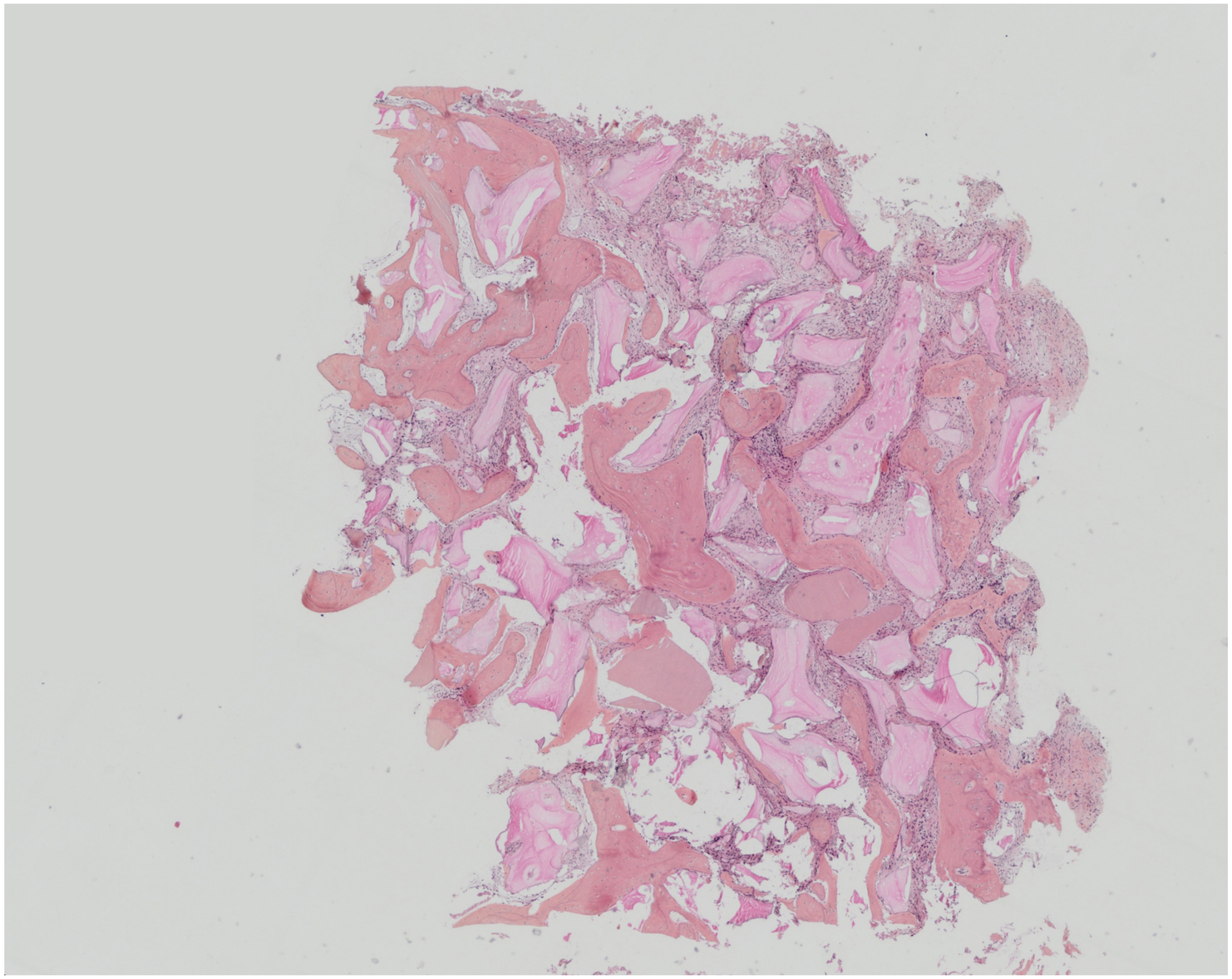


Total area	Bone area	Dentin area	Bio oss area	Sup Cal total	% Bone	% Dentin	% Bio oss	% Cal tot	% Partial bone	% Partial dentin	% Partial bio oss	1% Total area
898.893	306.707	115.556		422.263	34.121	12.855	0.000	46.976	72.634	27.366	0.000	0.111247946

Fig. 4-15 Sample I with its measurments

4.6 Samples of Test Group

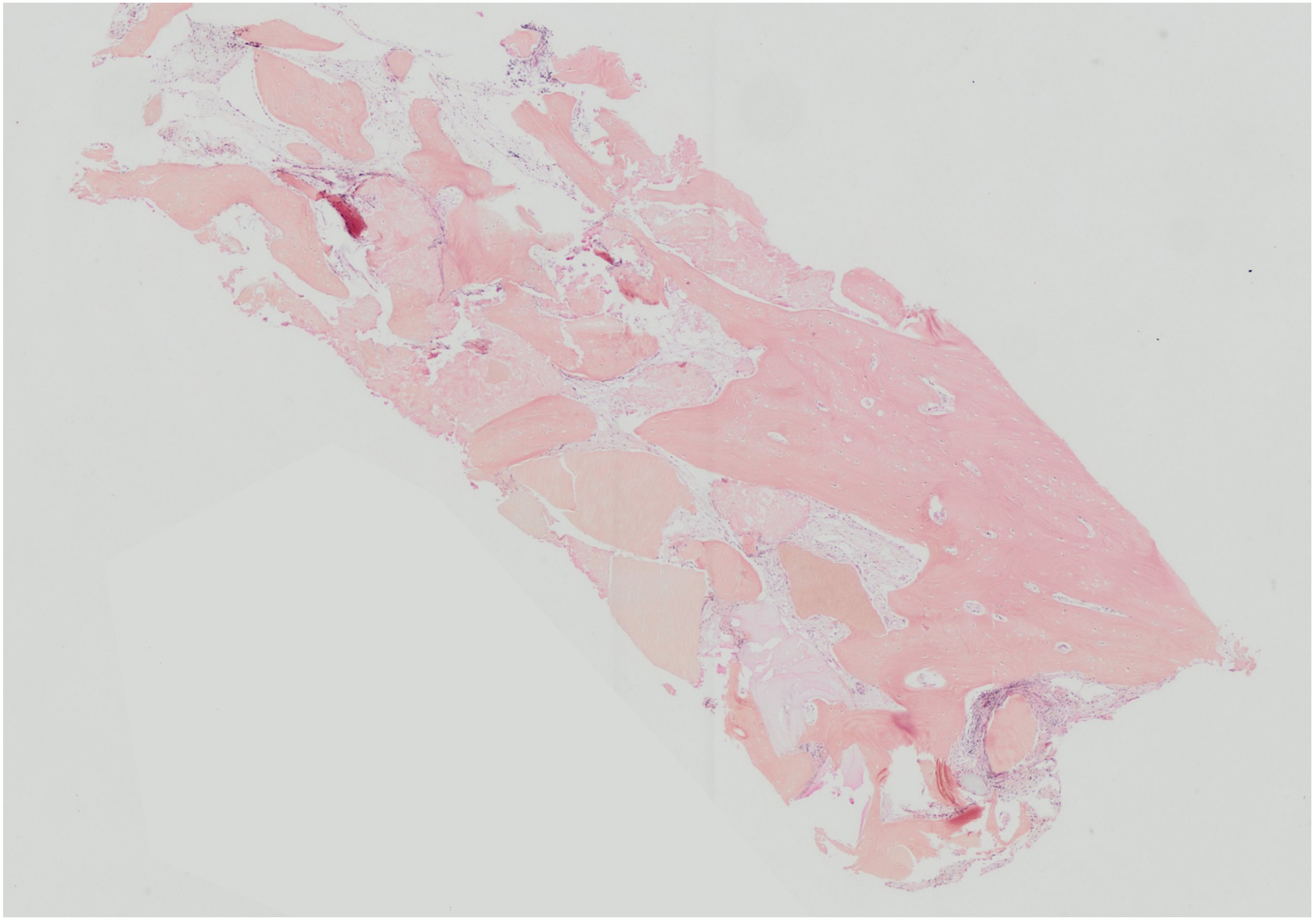
Sample 1



Total area	Bone area	Dentin area	Bio oss area	Sup Cal total	% Bone	% Dentin	% Bio oss	% Cal tot	% Partial bone	% Partial dentin	% Partial bio oss	1% Total area
1000.163	246.551	26.405	186.771	459.727	24.651	2.640	18.674	45.965	53.630	5.744	40.627	0.099983703

Fig. 4-16 Sample 1 with its measurments

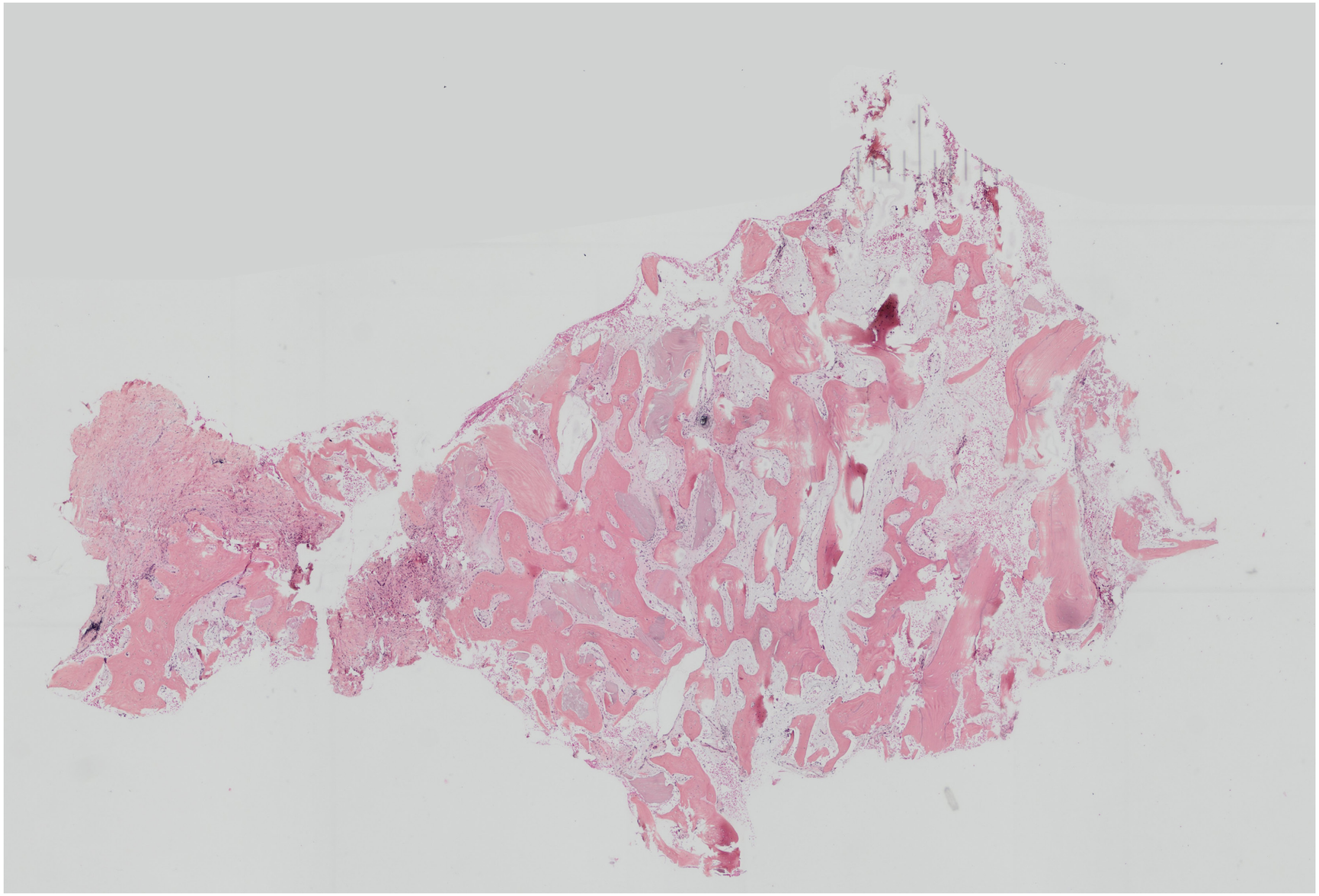
Sample 2



Total area	Bone area	Dentin area	Bio oss area	Sup Cal total	% Bone	% Dentin	% Bio oss	% Cal tot	% Partial bone	% Partial dentin	% Partial bio oss	1% Total area
453.57	198.399	30.256	9.535	238.19	43.742	6.671	2.102	52.514	83.294	12.702	4.003	0.220473135

Fig. 4-17 Sample 2 with its measurments

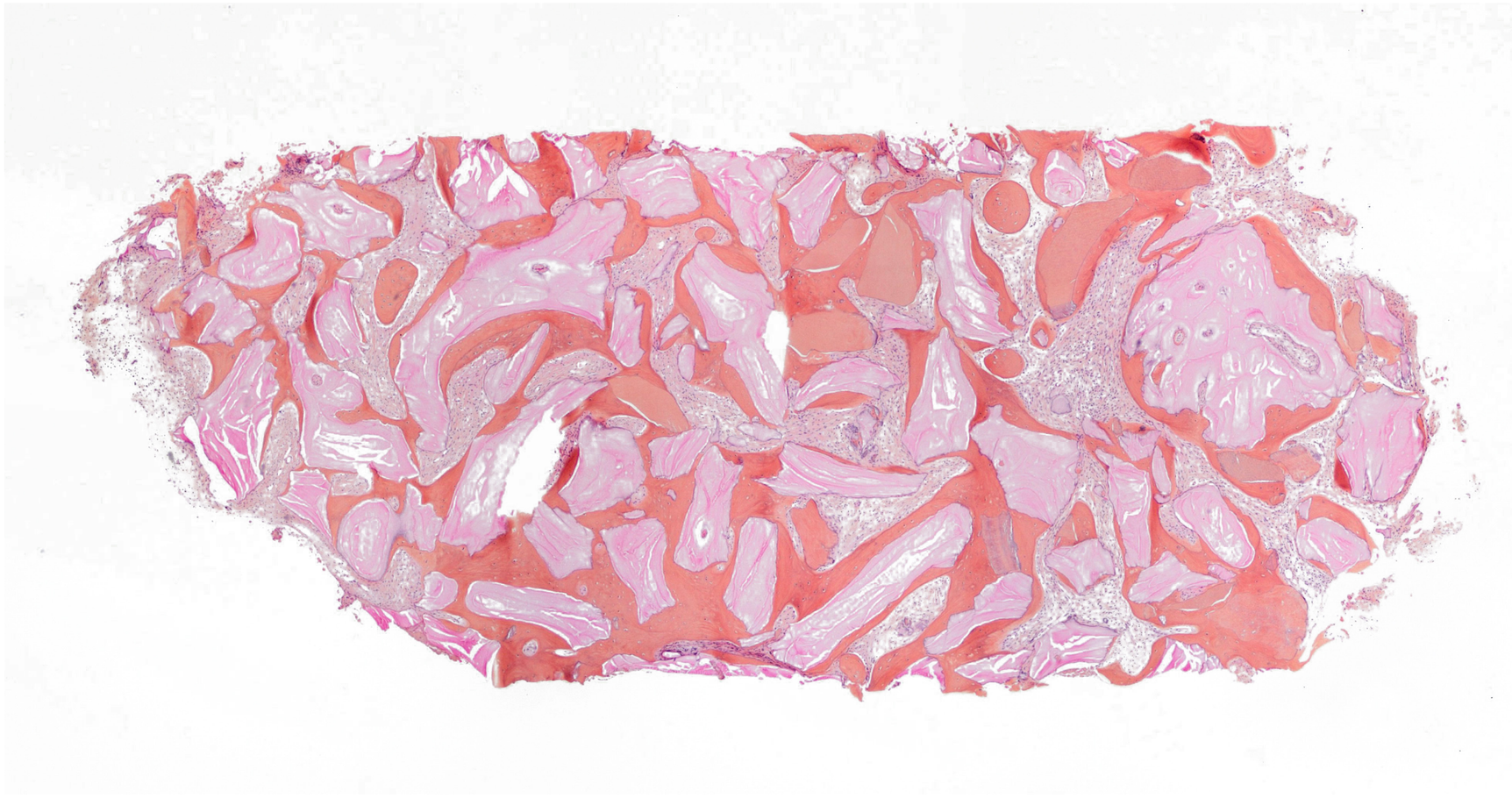
Sample 3



Total area	Bone area	Dentin area	Bio oss area	Sup Cal total	% Bone	% Dentin	% Bio oss	% Cal tot	% Partial bone	% Partial dentin	% Partial bio oss	1% Total area
1921.11	369.753	9.339	133.029	512.121	19.247	0.486	6.925	26.658	72.200	1.824	25.976	0.05205324

Fig. 4-18 Sample 3 with its measurments

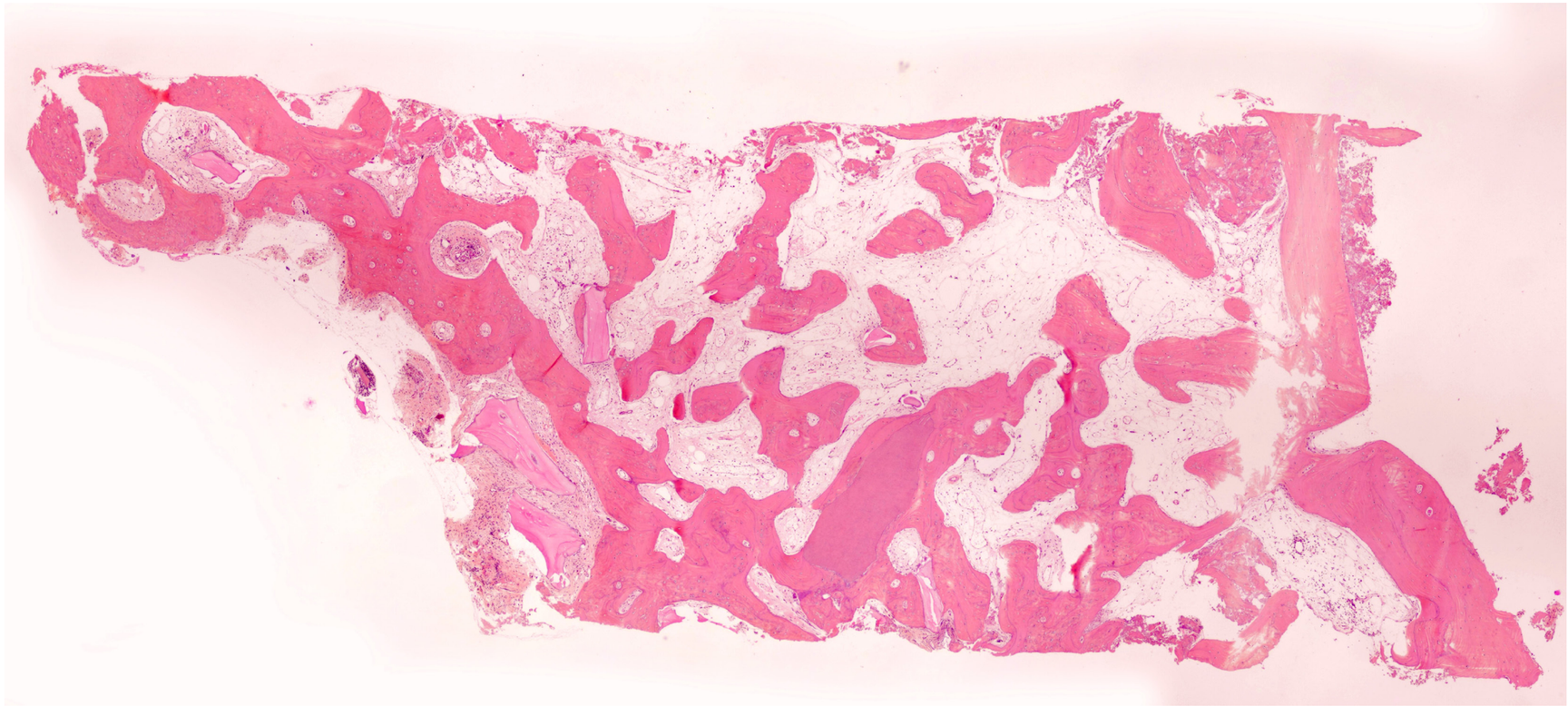
Sample 4



Total area	Bone area	Dentin area	Bio oss area	Sup Cal total	% Bone	% Dentin	% Bio oss	% Cal tot	% Partial bone	% Partial dentin	% Partial bio oss			1% Total area
797.628375	183.958	49.042125	243.814125	476.81425	23.42750582	5.972672	30.07099528	59.4711731	39.46080927	10.01913332	50.52005741		100	0.165938402

Fig. 4-19 Sample 4 with its measurments

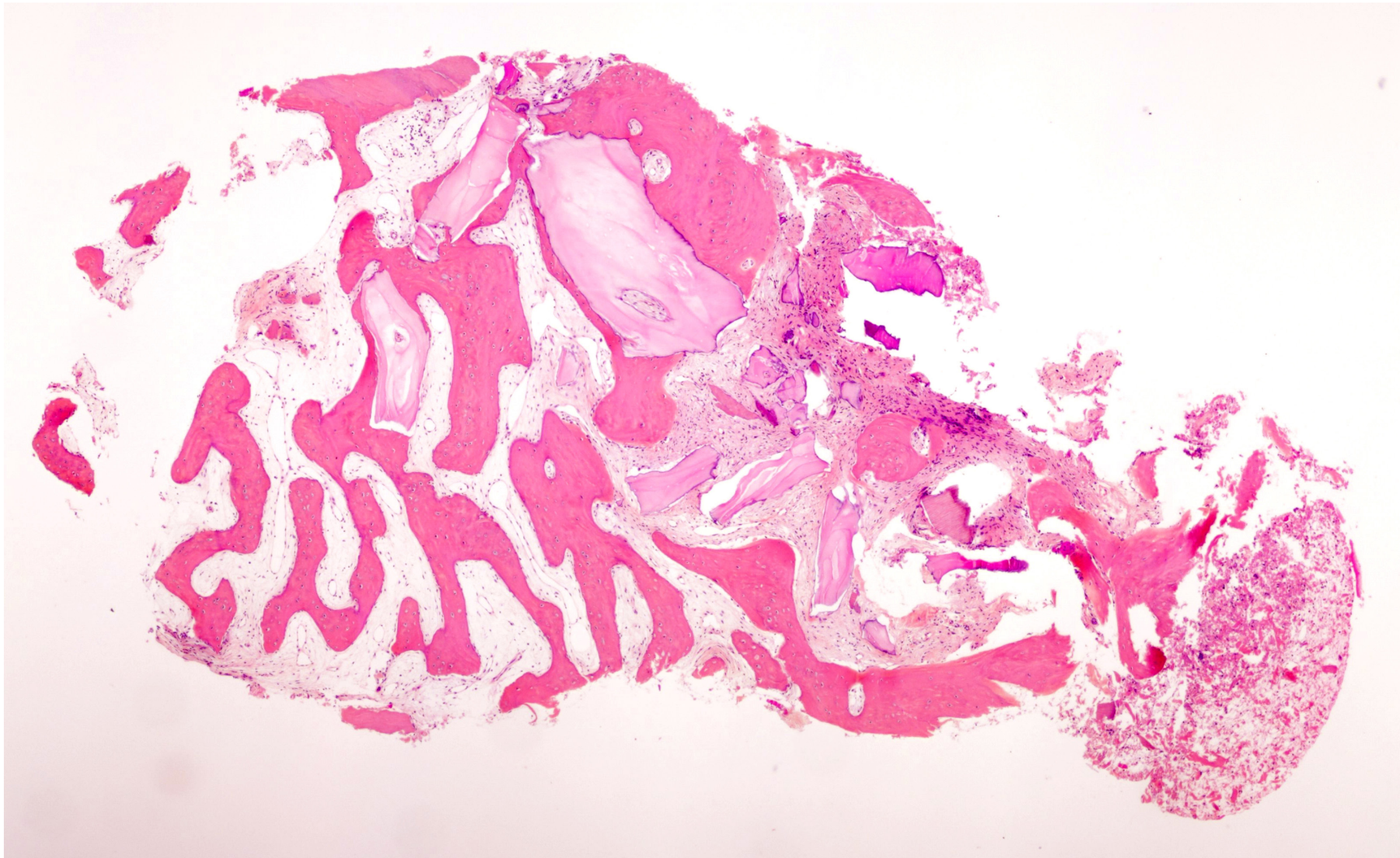
Sample 5



Total area	Bone area	Dentin area	Bio oss area	Sup Cal total	% Bone	% Dentin	% Bio oss	% Cal tot	% Partial bone	% Partial dentin	% Partial bio oss	1% Total area
1744.506	860.138	26.874	32.089	919.101	49.306	1.540	1.839	52.685	93.585	2.924	3.491	0.057322818

Fig. 4-20 Sample 5 with its measurments

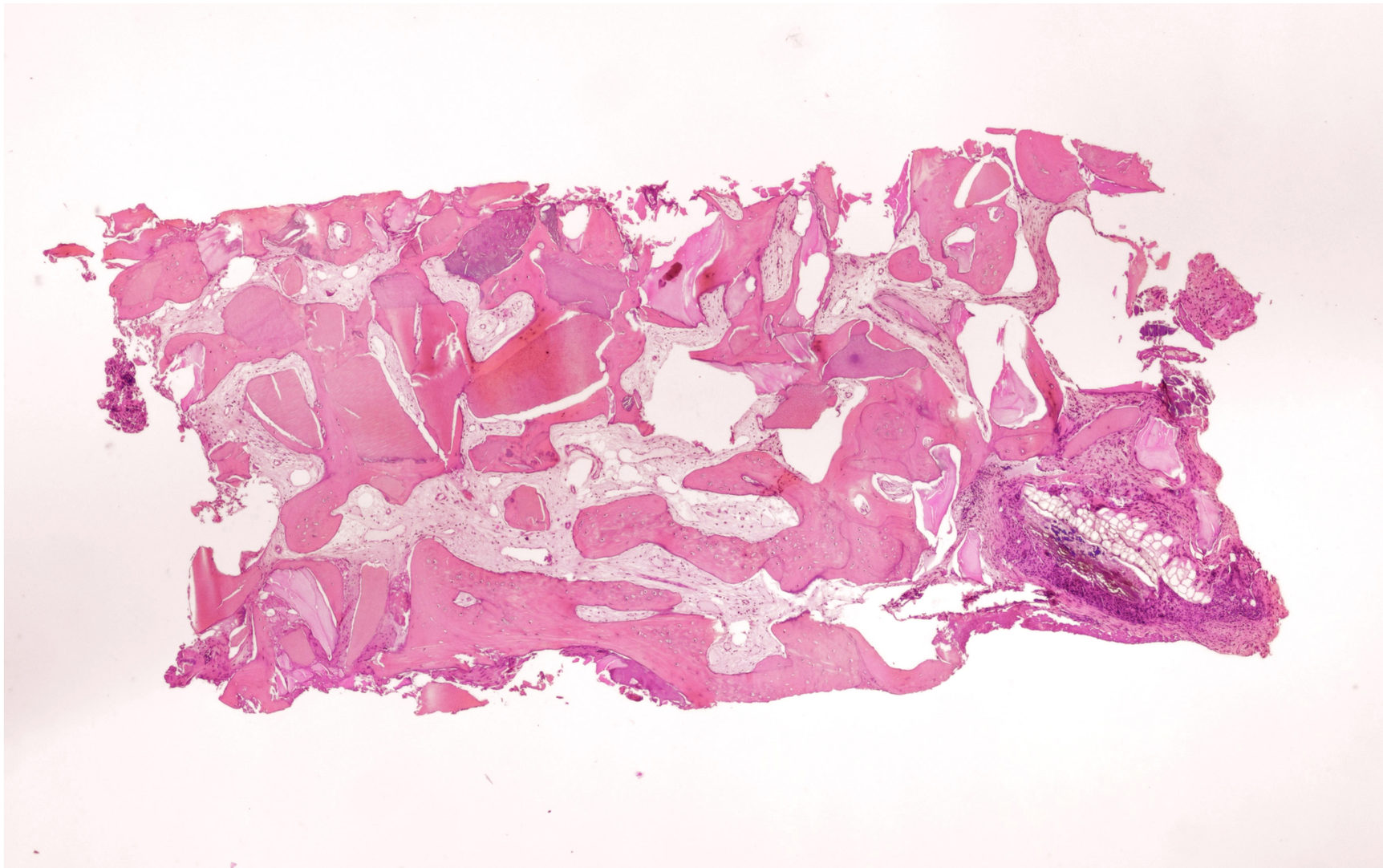
Sample 6



Total area	Bone area	Dentin area	Bio oss area	Sup Cal total	% Bone	% Dentin	% Bio oss	% Cal tot	% Partial bone	% Partial dentin	% Partial bio oss	1% Total area
525.467	146.788	5.922	44.224	196.934	27.935	1.127	8.416	37.478	74.537	3.007	22.456	0.190306908

Fig. 4-21 Sample 6 with its measurments

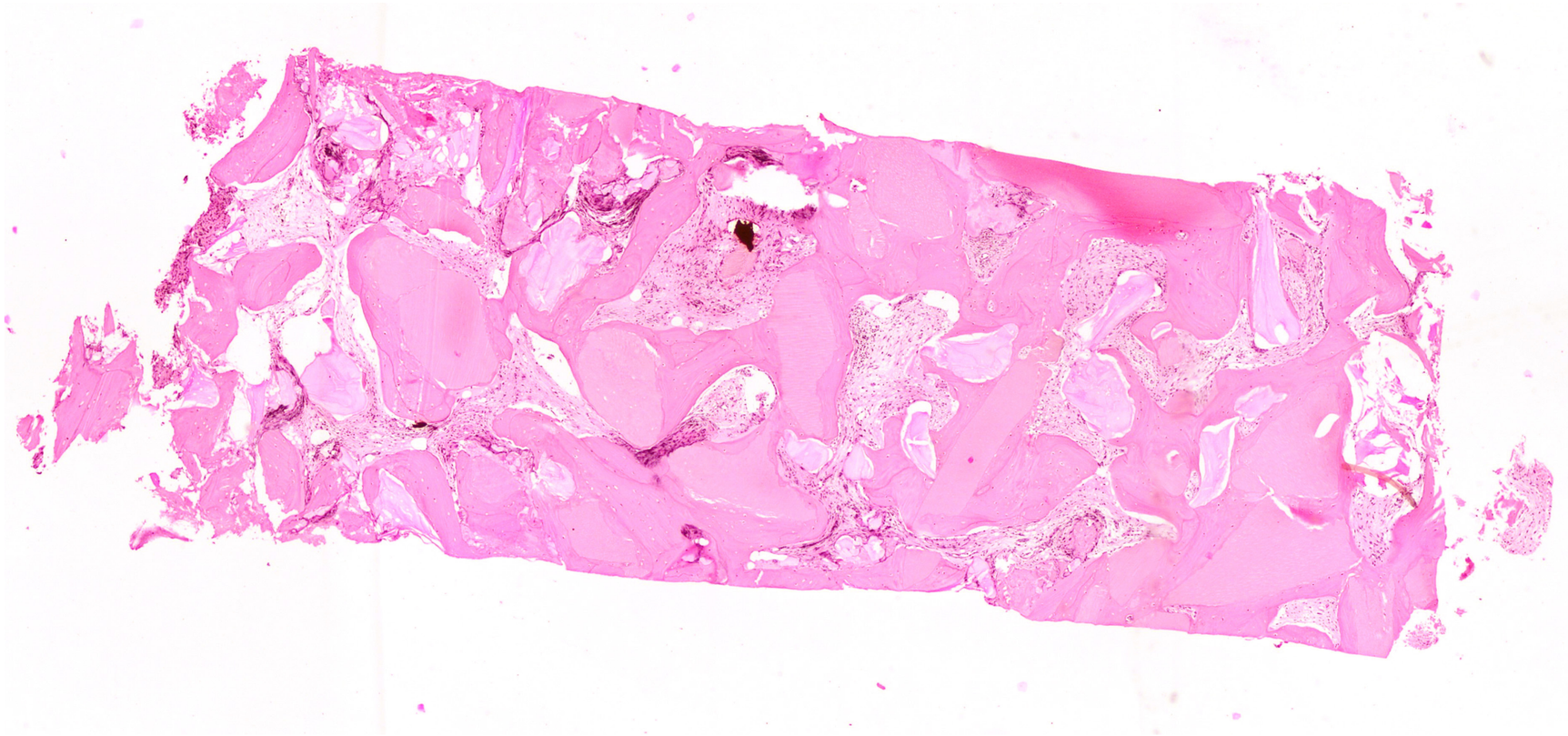
Sample 7



Total area	Bone area	Dentin area	Bio oss area	Sup Cal total	% Bone	% Dentin	% Bio oss	% Cal tot	% Partial bone	% Partial dentin	% Partial bio oss	1% Total area
589.566	164.491	39.771	22.611	226.873	27.900	6.746	3.835	38.481	72.504	17.530	9.966	0.169616294

Fig. 4-22 Sample 7 with its measurments

Sample 8



Total area	Bone area	Dentin area	Bio oss area	Sup Cal total	% Bone	% Dentin	% Bio oss	% Cal tot	% Partial bone	% Partial dentin	% Partial bio oss	1% Total area
800.844	273.945	136.199	55.294	465.438	34.207	17.007	6.904	58.118	58.857	29.263	11.880	0.124868264

Fig. 4-23 Sample 8 with its measurments

5 Results and Conclusion

5.1 Results of this study

A total of 17 samples from the test group and control group were analyzed. First of all, we calculated the amount of total mineralized tissue by adding the amount of bone area (mm), dentin area and Bio-Oss® area. Then we divided the area of the bone by the total area and multiplied the values by 100 to get the percentage of bone. And the same procedure for the percentages of dentin, Bio-Oss® and calcium total. Then, in order to determine the mineralized tissue fractions, the percentage of partial bone was calculated by division of bone area with total mineralized tissue multiple in 100 and the same procedures for the percentage of partial dentin and partial Bio-Oss® were done. (Table 5-1) As a statistical point of view, the mean, standard deviation (SD) and 95% confidence intervals were calculated for each experimental group. As mentioned before, one of the objectives of this study is to evaluate the amount of newly formed bone. So, by comparing the existing data, it is necessary to mention that the amount of newly formed bone was greater in the autogenous tooth matrix alone (control group) in comparison with the autogenous tooth matrix combined with Bio-Oss® (test group). (Table5-2).

	Mean	Standard Deviation
Control group	1456,64	691,91
Test group	305,50	235,29

Table 5-1 Amount of Bone Area (mm)

	Mean	Standard Deviation
Control group	36,33	5,63
Test group	31,30	10,43

Table 5-2 Amount of Bone Area (%)

Then we compared the amount of newly formed bone in percentage by creating a box and whiskers plot to know the distribution of data in terms of its median and quartiles in both groups. Before undertaking comparisons, the normality of all the distributions was tested with the Kolmogorov-Smirnov test, and the results were not significantly different from a normal distribution in all cases. Therefore, it was justified to use parametric tests like the unpaired Student's t-test for comparisons. A probability value P=0.05 was considered as the significance threshold.

From the box plot in (Fig 5-1) we can find that the amounts for the control group tend to be higher than for the test group. However, significance was not achieved (P=0.23), likely due to the relatively small sample size.

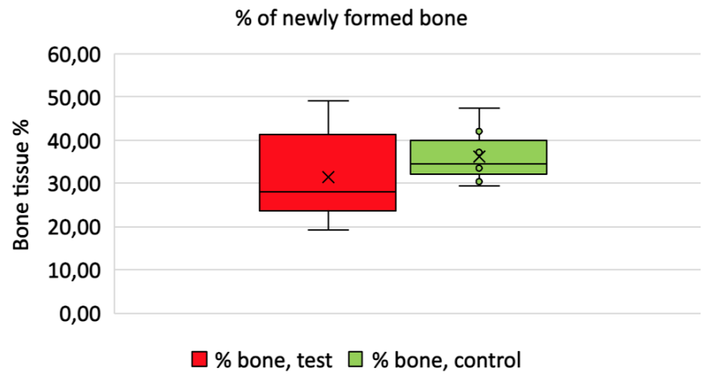


Fig. 5-1 Comparison of newly formed bone in Control and Test Groups

The distribution of data in both groups is not symmetrical, while the amount of new bone in the control group shows a higher median in comparison to the test group. The between-group comparison of total mineralized tissue and residual dentin was also performed. For the mineralized tissue (Fig. 5-2), the distribution of data in both groups is asymmetrical. The test group displayed a higher variability of mineralized tissue than the control group, as one can see on the whisker's plot. Also, the mean value for mineralized tissue in the test group shows a higher amount than in the control group. This is due to the residual presence of Bio-Oss that did not undergo resorption. However, also in this case, significance was not achieved (P=0.57). For residual dentin plot (Fig. 5-3), it is obvious that again in both groups there is a skewed distribution, but the control group displayed a higher median in comparison with the test group. This can be due

to the fact that a greater amount of dentin was placed in the defect site at surgery in the control group, as compared to the test, in which only 50% of the graft was dentin and the other 50% Bio-Oss®. Therefore, considering a similar resorption time of dentin in the two groups, a higher amount and % of dentin in the control group was expected.

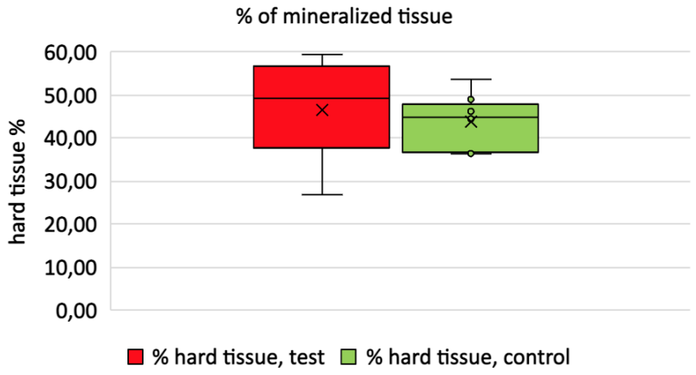


Fig. 5-2 Comparison of mineralized tissue in Control and Test Groups

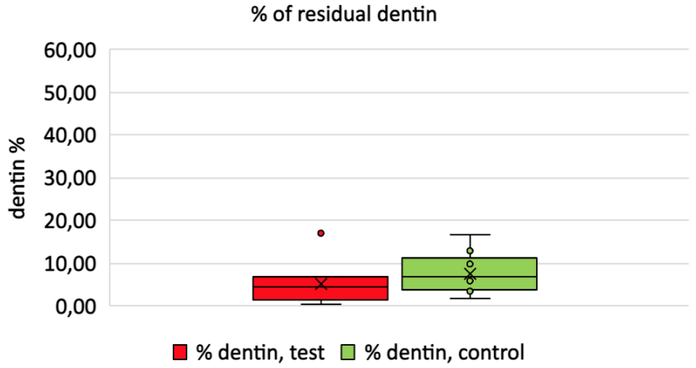


Fig. 5-3 Comparison of residual dentin in Control and Test Groups

In the next step we tried to evaluate the quality of the bone by analyzing the trabecular spaces (mm) in each sample. The

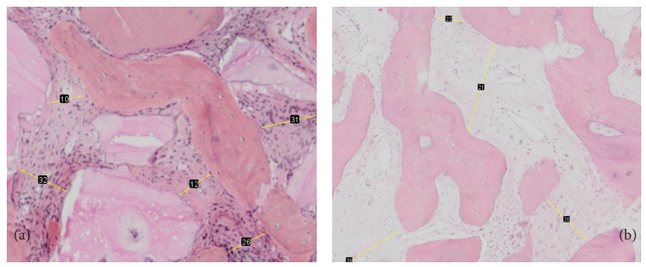


Fig. 5-4 Micrograph of test (a) and control (b) group
Yellow lines represent the random lines in soft tissue area

total trabecular space was calculated on ImageJ by considering 40 random lines in the soft tissue space between the bone, dentin, and Bio-Oss® areas in each sample (Fig. 5-4). After calculating the mean and standard deviation in each group, we found that in both groups there is not any variant in the data since SD is less than the mean value in both cases (Table 5-2). The between-group comparison gave a non-significant result (P=0.38). So, the compactness and quality of the bone tissue were found to be similar for the two groups. The lower number of trabecular spaces in the test group may also depend on the fact that 10% of the volume is still occupied by Bio-Oss.

	Mean	Standard Deviation
Control group	1.463111111	0.433379004
Test group	1.23275	0.621469169

Table 5-3 Comparison of Trabecular space data (mm)

In terms of comparison among mineralized data, we found the following data, which indicates that generally the bone areas in the control group are higher than in the test group, but there is not a significant difference between them. The total amount of autogenous mineralized tissue (new bone + dentin) was higher in the control group (43.87±6.11%) compared to the test group (36.58±12.48%), but the difference was not significant (P=0.14). Even if not statistically significant, there was a trend for greater new bone formation when using dentin graft alone as compared to the composite graft

	%Bone	%Dentin	% Bio-Oss®	%Auto hard tissue
Test group	31,30	5,27	9,85	36,58
Control group	36,33	7,54	0	43,87
SD Test group	10,43	5,39	9,76	
SD Control group	5,63	4,75	0	

Table 5-4 Comparison among mineralized fractions

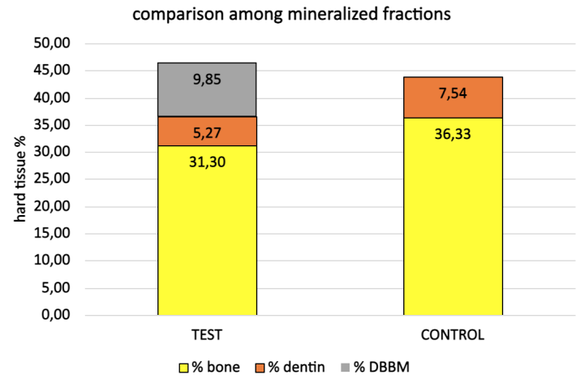


Fig. 5-5 Comparison among mineralized fractions

(36.33% vs. 31.30%), and a much reduced variability in results (standard deviation in the control group was about half that of the test group) (Table 5-4)(Fig.5-5).

5.2 Conclusion

Regarding the objectives of this study, we compare the amount of newly formed bone, mineralized tissue, residual dentin and the quality of bone in both groups. The amount of newly formed bone was greater in the autogenous tooth matrix alone (control group) in comparison with the autogenous tooth matrix combined with Bio-Oss® (test group). The interesting point in this case is that the control group shows a much smaller variation, as compared to the test group, meaning that the result can be more predictable when using tooth graft alone.

In the case of mineralized tissue, the mean value for mineralized tissue in the test group shows a higher amount than in the control group. This is because of the residual presence of Bio-Oss, which does not undergo resorption.

Moreover, residual dentin in the control group displayed a higher median in comparison with the test group due to the fact that a greater amount of dentin was placed in the defect site at surgery in the control group.

Analyzing the quality of the bone by analyzing the trabecular spaces (mm) allows us to know the compactness and quality of the bone tissue were found to be similar for the two groups. The lower trabecular spaces in the test group are due to the Bio-Oss, which occupied 10% of the volume.
The comparison among mineralized data shows that the total amount of autogenous mineralized tissue (new bone + dentin) was higher in the control group, but the difference was not significant. It is necessary to mention this fact that the non-significant difference in total mineralized tissues between the two groups is a positive result for the dentin graft. In fact, this would mean that using autogenous graft tissue alone would allow the patient to achieve similar mechanical support (e.g. in view of a future implant insertion) as using a mixture of dentin and Bio-Oss®, thereby reducing the financial cost for the patient and using a totally autogenous graft material. As mentioned before, even if not statistically

significant, there was a trend for greater new bone formation when using dentin graft alone as compared to the composite graft (36.33% vs. 31.30%), and a much reduced variability in results (SD in the control group was half of the test group). These too are positive results for dentin graft. The presence of two components in the graft, each with a different rate and mechanism of resorption, including a different angiogenesis pattern, may be a reason for the increased variability in stimulating the synthesis of new bone tissue.

5.3 Future work

Generally speaking, we discussed a lot of the problems regarding bone loss and related musculoskeletal problems that are created due to surgery or another reason, and it is obvious that many grafting substitutes have grown to overcome this challenge. While choosing an ideal material as a bone substitute is a difficult process, generally it is a fast and broad field for investigation. There is a possibility to overcome the limitations of the autologous bone grafting method by using bone tissue engineering, although choosing an optimized combination of the cells and scaffolds is still a challenge in this area. So, it is really important to choose an ideal material in terms of characteristics like mechanical properties, degradation rate, etc. Also, paying attention to the cost-effective and safe materials that lead to bone formation after bone graft implantation is a priority. According to the discussion, we believe that there are several potential ways to extend the aims of such a project. The suggestion in this case could be histomorphometric evaluation of autogenous grafts composed of only dentin versus different proportions of enamel and dentin, or doing a study to confirm tooth graft potential in different clinical applications, and finally, evaluating radiologically the long-term resorption of tooth grafts as compared to combined grafts or bio-oss alone.

6 Refrences

1. Prafulla K. Chandra, Shay Soker, Anthony Atala, Chapter 1 - Tissue engineering: current status and future perspectives, Editor(s): Robert Lanza, Robert Langer, Joseph P. Vacanti, Anthony Atala, Principles of Tissue Engineering (Fifth Edition), Academic Press, 2020, Page 2,

2. Chhibber, T.; Shinde, R.; Lahooti, B.; Bagchi, S.; Varahachalam, S.P.; Gaddam, A.; Jaiswal, A.K.; Gracia, E.;Chand, H.S.; Kaushik, A. Hydrogels in tissue engineering. InIntelligent Hydrogels in Diagnostics andTherapeutics; CRC Press: Boca Raton, FL, USA, 2020; pp. 105–106.

3. A. Khademhosseini, R.J.N.P. Langer, A decade of progress in tissue engineering, Nature protocols, 11 (2016): 1775.

4. Rider, Patrick, et al. "Bioprinting of Tissue Engineering Scaffolds." Journal of Tissue Engineering, Jan. 2018, doi:10.1177/2041731418802090.

5. Papaioannou, Theodore G et al. "3D Bioprinting Methods and Techniques: Applications on Artificial Blood Vessel Fabrication." Acta Cardiologica Sinica vol. 35,3 (2019): 284-289. doi:10.6515/ACS.201905_35(3).20181115A.

7. Cho DW, Kim BS, Jang J, Gao G, Han W, Singh N. 3D bioprinting: modeling in vitro tissues and organs using tissue-specific bioinks. 1st ed. Cham: Springer; 2019.

8. Madhusudan Singh; Hanna M. Haverinen; Parul Dhagat; Ghassan E. Jabbour (2010). Inkjet Printing–Process and Its Applications. , 22(6), 683.

9. Murphy, Sean V, and Anthony Atala. "3D bioprinting of tissues and organs." Nature biotechnology vol. 32,8 (2014): 776. doi:10.1038/nbt.2958.

10. Pepper, Matthew E et al. "Characterizing the effects of cell settling on bioprinter output." Biofabrication vol. 4,1 (2012): 011001. doi:10.1088/1758-5082/4/1/011001.

11. Tao Jiang, Jose G. Munguia-Lopez, Salvador Flores-Torres, Jacqueline Kort-Mascort, and Joseph M. Kinsella , "Extrusion bioprinting of soft materials: An emerging technique for biological model fabrication", Applied Physics Reviews 6, 011310 (2019) https://doi.org/10.1063/1.5059393.

12. Nguyen, Alexander K, and Roger J Narayan. "Liquid-Phase Laser Induced Forward Transfer for Complex Organic Inks and Tissue Engineering." Annals of biomedical engineering vol. 45,1 (2017): 84. doi:10.1007/s10439-016-1617-3.

13. Santoliquido, O., Camerota, F., Rosa, A., & Ortona, A. (2021). A novel device to simply 3D print bulk green ceramic components by stereolithography employing viscous slurries.

14. Jennifer J. Bara, Farshid Guilak, Chapter 10 - Engineering functional tissues: in vitro culture parameters, Editor(s): Robert Lanza, Robert Langer, Joseph P. Vacanti, Anthony Atala, Principles of Tissue Engineering (Fifth Edition), Academic Press, 2020, Page 157.

15. Hollister, Scott. (2005). Hollister, S.J.: Porous scaffold design for tissue engineering. Nat. Mater. 4, 518-524. Nature materials. 4. 518-24. 10.1038/nmat1421. AND Freed, Lisa E et al. "Advanced material strategies for tissue engineering scaffolds." Advanced materials (Deerfield Beach, Fla.) vol. 21,32-33 (2009): 3410-8. doi:10.1002/adma.200900303.

16. Nikolova, Maria P, and Murthy S Chavali. "Recent advances in biomaterials for 3D scaffolds: A review." Bioactive materials vol. 4 271-292. 25 Oct. 2019, doi:10.1016/j.bioactmat.2019.10.005.

17. Kurniawan, Nicholas A. "The ins and outs of engineering functional tissues and organs: evaluating the in-vitro and in-situ

processes." Current opinion in organ transplantation vol. 24,5 (2019): 595. doi:10.1097/MOT.0000000000000690.

18.1. Muschler GF, Nakamoto C, Griffith LG. Engineering principles of clinical cell-based tissue engineering. J Bone Joint Surg Am 2004;86-A(7):154158.
18.2. Yves Martin, Patrick Vermette, Bioreactors for tissue mass culture: Design, characterization, and recent advances, Biomaterials, Volume 26, Issue 35, 2005, Pages 7481-7503,.
18.3. Ivan Martin, David Wendt, Michael Heberer, The role of bioreactors in tissue engineering, Trends in Biotechnology, Volume 22, Issue 2, 2004, Pages 80-86.
18.4. Radisic M, Marsano A, Maidhof R, Wang Y, Vunjak-Novakovic G. Cardiac tissue engineering using perfusion bioreactor systems. Nat Protoc 2008;3(4):71938.
18.5. Wendt D, Riboldi SA, Cioffi M, Martin I. Potential and bottlenecks of bioreactors in 3D cell culture and tissue manufacturing. Adv Mater 2009;21(3233):335267.
18.6. Tandon N, Cannizzaro C, Chao PH, Maidhof R, Marsano A, Au HT, et al. Electrical stimulation systems for cardiac tissue engineering. Nat Protoc 2009;4(2):15573.
18.7. Iyer RK, Chiu LL, Reis LA, Radisic M. Engineered cardiac tissues. Curr Opin biotechnology 2011;22(5):70614.

19. Hanry Yu, Seow Khoon Chong, Ammar Mansoor Hassanbhai, Yao Teng, Gowri Balachander, Padmalosini Muthukumaran, Feng Wen, Swee Hin Teoh, Chapter 11 - Principles of bioreactor design for tissue engineering, Editor(s): Robert Lanza, Robert Langer, Joseph P. Vacanti, Anthony Atala, Principles of Tissue Engineering (Fifth Edition), Academic Press, 2020.

20. Kristen L. Moffat, Rebekah A. Neal, Lisa E. Freed, Farshid Guilak, Chapter 13 - Engineering Functional Tissues: In Vitro Culture Parameters, Editor(s): Robert Lanza, Robert Langer, Joseph Vacanti, Principles of Tissue Engineering (Fourth Edition), Academic Press, 2014, Page 159.

21.

22. V. Prasad Shastri, Chapter 15 - In vivo engineering of organs, Editor(s): Robert Lanza, Robert Langer, Joseph P. Vacanti, Anthony Atala, Principles of Tissue Engineering (Fifth Edition), Academic Press, 2020, Pages 261-262.

23. Ravaglioli A, Krawjesky R. Bioceramics. Netherlands: Springer; 1992. ISBN 978-94-010-5032-6.

24. Dolcimascolo, Anna & Calabrese, Giovanna & Conoci, Sabrina & Parenti, Rosalba. (2019). Innovative Biomaterials for Tissue Engineering. 10.5772/intechopen.83839.

25. Fergal J. O'Brien, Biomaterials & scaffolds for tissue engineering, Materials Today, Volume 14, Issue 3, 2011, Pages 88-95.

26. Fergal J. O'Brien, Biomaterials & scaffolds for tissue engineering, Materials Today, Volume 14, Issue 3, 2011, Pages 88-95.

27. Ige, O.O., Umoru, L.E., Aribo, S., 2012. Natural Products: A Minefield of Biomaterials. ISRN Materials Science 2012, 1–20. doi:10.5402/2012/983062.

28. Ha, Tran & Minh, To & Nguyen, Doan & Minh, Do. (2013). Naturally Derived Biomaterials: Preparation and Application. doi: 10.5772/55668.

29. Jenkins, Cara L et al. "Effect of 3-hydroxyproline residues on collagen stability." Journal of the American Chemical Society vol. 125,21 (2003): 6422-7. doi:10.1021/ja034015j.

30. Rebecca A. MacDonald, Christopher M. Voge, Mihalis Kariolis, Jan P. Stegemann, Carbon nanotubes increase the electrical conductivity of fibroblast-seeded collagen hydrogels, Acta Biomaterialia, Volume 4, Issue 6, 2008, Pages 1583-1592.

31. Ige, O.O., Umoru, L.E., Aribo, S., 2012. Natural Products: A Minefield of Biomaterials. ISRN Materials Science 2012, 1–20. doi:10.5402/2012/983062.

32. Frank-Kamenetskaya, Olga & Vlasov, Dmitrii & Panova, Elena & Lessovaia, Sofia. (2020). Processes and Phenomena

on the Boundary Between Biogenic and Abiogenic Nature. 10.1007/978-3-030-21614-6.

33. Stephen F. Badylak, Xenogeneic extracellular matrix as a scaffold for tissue reconstruction, Transplant Immunology, Volume 12, Issues 3–4, 2004, Pages 367-377.

34. Farokhi, M., Farinaz Jonidi Shariatzadeh, A. Solouk and H. Mirzadeh. "Alginate Based Scaffolds for Cartilage Tissue Engineering: A Review." International Journal of Polymeric Materials and Polymeric Biomaterials 69 (2020): 230 - 247.

35. O.S. Manoukian, A. Ahmad, C. Marin, R. James, A.D. Mazzocca, S.G. Kumbar, 22 - Bioactive nanofiber dressings for wound healing, Editor(s): Magnus S. Ågren, Wound Healing Biomaterials, Woodhead Publishing, 2016, Pages 451-481.

36. Ohan S. Manoukian, Naseem Sardashti, Teagen Stedman, Katie Gailiunas, Anurag Ojha, Aura Penalosa, Christopher Mancuso, Michelle Hobert, Sangamesh G. Kumbar, Biomaterials for Tissue Engineering and Regenerative Medicine, Editor(s): Roger Narayan, Encyclopedia of Biomedical Engineering, Elsevier, 2019, Pages 462-482.

37. Andre L. Rossi, Isabela C. Barreto, William Q. Maciel, Fabiana P. Rosa, Maria H. Rocha-Leão, Jacques Werckmann, Alexandre M. Rossi, Radovan Borojevic, Marcos Farina, Ultrastructure of regenerated bone mineral surrounding hydroxyapatite–alginate composite and sintered hydroxyapatite, Bone, Volume 50, Issue 1, 2012, Pages 301-310.

38. Grulova, I et al. "Delivery of Alginate Scaffold Releasing Two Trophic Factors for Spinal Cord Injury Repair." Scientific reports vol. 5 13702. 8 Sep. 2015, doi:10.1038/srep13702.

39. Sultankulov, Bolat, Dmitriy Berillo, Karina Sultankulova, Tursonjan Tokay, and Arman Saparov. 2019. "Progress in the Development of Chitosan-Based Biomaterials for Tissue Engineering and Regenerative Medicine" Biomolecules 9, no. 9: 470. https://doi.org/10.3390/biom9090470.

40. Dutta, P. et al. "Chitin and chitosan: Chemistry, properties and applications." Journal of Scientific & Industrial Research 63 (2004): 20-31.

41. Kundu, B., Rajkhowa, R., Kundu, S. C., & Wang, X. (2013). Silk fibroin biomaterials for tissue regenerations. Advanced drug delivery reviews, 65(4), 457–470. https://doi.org/10.1016/j.addr.2012.09.043.

42. S.K. Jindal, M. Kiamehr, W. Sun, X.B. Yang, 15 - Silk scaffolds for dental tissue engineering, Editor(s): S.C. Kundu, Silk Biomaterials for Tissue Engineering and Regenerative Medicine, Woodhead Publishing, 2014, Pages 403-428.

43. S. Das, U. Bora, B.B. Borthakur,. "Applications of silk biomaterials in tissue engineering and regenerative medicine"; Silk Biomaterials for Tissue Engineering and Regenerative Medicine, S.C. Kundu, Woodhead Publishing, 2014, Pages 41-77.

44.1. Domingues, R. M., Gomes, M. E., & Reis, R. L. (2014). The potential of cellulose nanocrystals in tissue engineering strategies. Biomacromolecules, 15(7), 2327–2346. https://doi.org/10.1021/bm500524s.
44.2. Mohite, B.V. and Patil, S.V. (2014), A novel biomaterial: bacterial cellulose and its new era applications. Biotechnology and Applied Biochemistry, 61: 101-110. https://doi.org/10.1002/bab.1148.
44.3. Courtenay, James C., Ram I. Sharma, and Janet L. Scott 2018. "Recent Advances in Modified Cellulose for Tissue Culture Applications" Molecules 23, no. 3: 654. https://doi.org/10.3390/molecules23030654.

45. Hickey, R. J., & Pelling, A. E. (2019). Cellulose Biomaterials for Tissue Engineering. Frontiers in bioengineering and biotechnology, 7, 45. https://doi.org/10.3389/fbioe.2019.00045.

46.1. Innala, Marcus et al. "3D culturing and differentiation of SH-SY5Y neuroblastoma cells on bacterial nanocellulose scaffolds." Artificial cells, nanomedicine, and biotechnology vol. 42,5 (2014): 302-8. doi:10.3109/21691401.2013.821410.
46.2. Jonsson, M., Brackmann, C., Puchades, M., Brattås, K., Ewing, A., Gatenholm, P., & Enejder, A. (2015). Neuronal Networks on Nanocellulose Scaffolds. Tissue engineering. Part C, Methods, 21(11), 1162–1170. https://doi.org/10.1089/ten.tec.2014.0602.

47.1. Makogonenko, E., Tsurupa, G., Ingham, K., & Medved, L. (2002). Interaction of fibrin(ogen) with fibronectin: further characterization and localization of the fibronectin-binding site. Biochemistry, 41(25), 7907–7913. https://doi.org/10.1021/bi025770x.
47.2. Rybarczyk, B. J., Lawrence, S. O., & Simpson-Haidaris, P. J. (2003). Matrix-fibrinogen enhances wound closure by increasing both cell proliferation and migration. Blood, 102(12), 4035–4043. https://doi.org/10.1182/blood-2003-03-0822 .
47.3. Mosesson M. W. (2005). Fibrinogen and fibrin structure and functions. Journal of thrombosis and haemostasis : JTH, 3(8), 1894–1904. https://doi.org/10.1111/j.1538-7836.2005.01365.x.
47.4. Laurens, N., Koolwijk, P., & de Maat, M. P. (2006). Fibrin structure and wound healing. Journal of thrombosis and haemostasis : JTH, 4(5), 932–939. https://doi.org/10.1111/j.1538-7836.2006.01861.x.
47.5. Wolberg A. S. (2007). Thrombin generation and fibrin clot structure. Blood reviews, 21(3), 131–142. https://doi.org/10.1016/j.blre.2006.11.001.

48.1. Martino, M. M., Mochizuki, M., Rothenfluh, D. A., Rempel, S. A., Hubbell, J. A., & Barker, T. H. (2009). Controlling integrin specificity and stem cell differentiation in 2D and 3D environments through regulation of fibronectin domain stability. Biomaterials, 30(6), 1089–1097. https://doi.org/10.1016/j.biomaterials.2008.10.047.
48.2. Catelas, I., Sese, N., Wu, B. M., Dunn, J. C., Helgerson, S., & Tawil, B. (2006). Human mesenchymal stem cell proliferation and osteogenic differentiation in fibrin gels in vitro. Tissue engineering, 12(8), 2385–2396. https://doi.org/10.1089/ten.2006.12.2385.
48.3. Zhang, G., Wang, X., Wang, Z., Zhang, J., & Suggs, L. (2006). A PEGylated fibrin patch for mesenchymal stem cell delivery. Tissue engineering, 12(1), 9–19. https://doi.org/10.1089/ten.2006.12.9 AND Huang, N. F., Lam, A., Fang, Q., Sievers, R. E., Li, S., & Lee, R. J. (2009). Bone marrow-derived mesenchymal stem cells in fibrin augment angiogenesis in the chronically infarcted myocardium. Regenerative medicine, 4(4), 527–538. https://doi.org/10.2217/rme.09.32.
48.4. Takei, A., Tashiro, Y., Nakashima, Y., & Sueishi, K. (1995). Effects of fibrin on the angiogenesis in vitro of bovine endothelial cells in collagen gel. In vitro cellular & developmental biology. Animal, 31(6), 467–472. https://doi.org/10.1007/BF02634260.

49. Spotnitz, W.D. Fibrin Sealant: Past, Present, and Future: A Brief Review. World J Surg 34, 632–634 (2010). https://doi.org/10.1007/s00268-009-0252-7.

50. Maria Luísa Leite, Diana Gabriela Soares, Giovana Anovazzi, Caroline Anselmi, Josimeri Hebling, Carlos Alberto de Souza Costa. (2021). "Fibronectin-loaded Collagen/Gelatin Hydrogel Is a Potent Signaling Biomaterial for Dental Pulp Regeneration"; Journal of Endodontics, 47(7), 1110-1117. DOI: 10.22203/ecm.v008a01

51. Ashley Thomas & Japes Bera (2019) Preparation and characterization of gelatin-bioactive glass ceramic scaffolds for bone tissue engineering, Journal of Biomaterials Science, Polymer Edition, 30:7, 561-579, DOI: 10.1080/09205063.2019.1587697.

52. Alvin Bacero Bello, Deogil Kim, Dohyun Kim, Hansoo Park, and Soo-Hong Lee, (2020). "Engineering and Functionalization of Gelatin Biomaterials: From Cell Culture to Medical Applications"; Tissue Engineering Part B: Reviews, 26(2), 164-180. https://doi.org/10.1089/ten.teb.2019.0256.

53. Selestina Gorgieva and Vanja Kokol (November 16th 2011). Collagen- vs. Gelatine-Based Biomaterials and Their Biocompatibility: Review and Perspectives, Biomaterials Applications for Nanomedicine, Rosario Pignatello, IntechOpen, DOI: 10.5772/24118. Available from: https://www.intechopen.com/chapters/23617.

54. Seblewongel Petros, Tamrat Tesfaye, Million Ayele, "A Review on Gelatin Based Hydrogels for Medical Textile Applications", Journal of Engineering, vol. 2020, Article ID 8866582, 12 pages, 2020. https://doi.org/10.1155/2020/8866582.

55. Maria Luísa Leite, Diana Gabriela Soares, Giovana Anovazzi, Caroline Anselmi, Josimeri Hebling, Carlos Alberto de Souza Costa. (2021), "Fibronectin-loaded Collagen/Gelatin Hydrogel Is a Potent Signaling Biomaterial for Dental Pulp Regeneration"; Journal of Endodontics, 47 (7), 1110-1117. https://doi.org/10.1016/j.joen.2021.04.009.

56. Ohan S. Manoukian, Naseem Sardashti, Teagen Stedman, Katie Gailiunas, Anurag Ojha, Aura Penalosa, Christopher

Mancuso, Michelle Hobert, Sangamesh G. Kumbar. "Biomaterials for Tissue Engineering and Regenerative Medicine", Editor(s): Roger Narayan, Encyclopedia of Biomedical Engineering, Elsevier, 2019, 462-482, https://doi.org/10.1016/B978-0-12-801238-3.64098-9.

57. Ulery, B.D., Nair, L.S. and Laurencin, C.T. (2011), Biomedical applications of biodegradable polymers. J. Polym. Sci. B Polym. Phys., 49: 832-864. https://doi.org/10.1002/polb.22259.

58. Anderson M, et al. (2015) Peripheral nerve regeneration strategies: Electrically stimulating polymer based nerve growth conduits. Critical Reviews™in Biomedical Engineering 43: 2–3.

59. Sakaguchi Y, et al. (2015) Polyglycolic acid sheets with fibrin glue can prevent esophageal stricture after endoscopic submucosal dissection. Endoscopy 47(04): 336–340.

60. Stratton, S., Manoukian, O. S., Patel, R., Wentworth, A., Rudraiah, S. and Kumbar, S. G. (2018), Polymeric 3D printed structures for soft-tissue engineering. J. Appl. Polym. Sci., 135, 45569. doi: 10.1002/app.45569.

61. John C Middleton, Arthur J Tipton, (2000). "Synthetic biodegradable polymers as orthopedic devices"; Biomaterials, 21(23), 2335-2346. https://doi.org/10.1016/S0142-9612(00)00101-0.

62. Casalini Tommaso, Rossi Filippo, Castrovinci Andrea, Perale Giuseppe, (2019). "A Perspective on Polylactic Acid-Based Polymers Use for Nanoparticles Synthesis and Applications"; Frontiers in Bioengineering and Biotechnology, 7, 259. DOI=10.3389/fbioe.2019.00259.

63. Makadia, Hirenkumar K., and Steven J. Siegel 2011. "Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier" Polymers 3, no. 3: 1377-1397. https://doi.org/10.3390/polym3031377.

64. Philipp Stockmann, Hartmut Böhm, Oliver Driemel, Joachim Mühling, Hans Pistner. (2010). "Resorbable versus titanium osteosynthesis devices in bilateral sagittal split ramus

osteotomy of the mandible – the results of a two centre randomised clinical study with an eight-year follow-up"; Journal of Cranio-Maxillofacial Surgery, 38(7), 522-528. https://doi.org/10.1016/j.jcms.2010.01.002.

65. Selim, M., Bullock, A.J., Blackwood, K.A., Chapple, C.R. and MacNeil, S. (2011), Developing biodegradable scaffolds for tissue engineering of the urethra. BJU International, 107: 296-302. https://doi.org/10.1111/j.1464-410X.2010.09310.x.

66. Elbay Malikmammadov, Tugba Endogan Tanir, Aysel Kiziltay, Vasif Hasirci & Nesrin Hasirci (2018) PCL and PCL-based materials in biomedical applications, Journal of Biomaterials Science, Polymer Edition, 29:7-9, 863-893, DOI: 10.1080/09205063.2017.1394711.

67. Manoukian OS, et al. (2016b) Tissue-engineered medical products. In: Biomaterials and nanotechnology for tissue engineering, 289 pp.

68. M.J. Roberts, M.D. Bentley, J.M. Harris. (2002). "Chemistry for peptide and protein PEGylation"; Advanced Drug Delivery Reviews, 54(4), 459-476. https://doi.org/10.1016/S0169-409X(02)00022-4.

69. Alcantar NA, Aydil ES, and Israelachvili JN (2000) Polyethylene glycol-coated biocompatible surfaces. Journal of Biomedical Materials Research 51: 343–351.

70. Pielichowski K and Njuguna J (2005) Thermal degradation of polymeric materials. iSmithers Rapra Publishing.

71. Hosseinpour, Sepanta & Walsh, Laurence & Moharamzadeh, Keyvan. (2021). Regenerative Approaches in Dentistry.

72. Zheng, C., Chen, J., Liu, S., & Jin, Y. (2019). Stem cell-based bone and dental regeneration: a view of microenvironmental modulation. International journal of oral science, 11(3), 23. https://doi.org/10.1038/s41368-019-0060-3.

73. Wang, J., Jiao, D., Huang, X. et al. Osteoclastic effects of mBMMSCs under compressive pressure during orthodontic tooth movement. Stem Cell Res Ther 12, 148 (2021). https://doi.org/10.1186/s13287-021-02220-0.

74. Liu Y and Holmes C (2021) Tissue Regeneration Capacity of Extracellular Vesicles Isolated From Bone Marrow-Derived and Adipose-Derived Mesenchymal Stromal/ Stem Cells. Front. Cell Dev. Biol. 9:648098. doi: 10.3389/ fcell.2021.648098.

75. Gardin, Chiara & Ricci, Sara & Ferroni, Letizia. (2016). Dental Stem Cells (DSCs): Classification and Properties. doi:10.1007/978-3-319-33299-4_1.

76. Gardin C., Ricci S., Ferroni L. (2016) Dental Stem Cells (DSCs): Classification and Properties. In: Zavan B., Bressan E. (eds) Dental Stem Cells: Regenerative Potential. Stem Cell Biology and Regenerative Medicine. Humana Press, Cham. https://doi.org/10.1007/978-3-319-33299-4_1.

77. Shi, X, Mao, J, Liu, Y. Pulp stem cells derived from human permanent and deciduous teeth: Biological characteristics and therapeutic applications. STEM CELLS Transl Med. 2020; 9: 445– 464. https://doi.org/10.1002/sctm.19-0398.

78. Geetha Manivasagam, Aakash Reddy, Dwaipayan Sen, Sunita Nayak, Mathew T. Mathew, Asokami Rajamanikam, Dentistry: Restorative and Regenerative Approaches, Editor(s): Roger Narayan, Encyclopedia of Biomedical Engineering, Elsevier, 2019, Pages 332-347.

79. Wang, D., Wang, Y., Tian, W., & Pan, J. (2019). Advances of tooth-derived stem cells in neural diseases treatments and nerve tissue regeneration. Cell proliferation, 52(3), e12572. https://doi.org/10.1111/cpr.12572.

80. Tatic, N., Rose, F.R.A.J., des Rieux, A. et al. Stem cells from the dental apical papilla in extracellular matrix hydrogels mitigate inflammation of microglial cells. Sci Rep 9, 14015 (2019). https://doi.org/10.1038/s41598-019-50367-x.

81. Gamilah Al-Qadhi, Malak Soliman, Iman Abou-Shady, Laila Rashed. (2020).“Gingival mesenchymal stem cells as an alternative source to bone marrow mesenchymal stem cells in regeneration of bone defects: In vivo study”; Tissue and Cell, Volume 63. https://doi.org/10.1016/j.tice.2019.101325.

82. Cristaldi M, Mauceri R, Campisi G, Pizzo G, Alessandro R, Tomasello L, Pitrone M, Pizzolanti G and Giordano C (2020) Growth and Osteogenic Differentiation of Discarded Gingiva-Derived Mesenchymal Stem Cells on a Commercial Scaffold. Front. Cell Dev. Biol. 8:292. doi: 10.3389/ fcell.2020.00292.

83. Suchánek J, Browne KZ, Nasry SA, Kleplová TS, Pilbauerová N, Schmidt J, Soukup T. Characteristics of Human Natal Stem Cells Cultured in Allogeneic Medium. Braz Dent J. 2018 Sep-Oct;29(5):427-434. doi: 10.1590/0103-6440201802388. PMID: 30517440.

84. Kumar, Prasanna et al. “Bone grafts in dentistry.” Journal of pharmacy & bioallied sciences vol. 5,Suppl 1 (2013): S125-7. doi:10.4103/0975-7406.113312.

85. Zeeshan Sheikh, Nader Hamdan, Mohamed-Nur Abdallah, Michael Glogauer, Marc Grynpas, 15 - Natural and synthetic bone replacement graft materials for dental and maxillofacial applications, Editor(s): Zohaib Khurshid, Shariq Najeeb, Muhammad Sohail Zafar, Farshid Sefat, Advanced Dental Biomaterials, Woodhead Publishing, 2019, Pages 347-376.

86. Dias, Rafael R et al. “Corticocancellous fresh-frozen allograft bone blocks for augmenting atrophied posterior mandibles in humans.” Clinical oral implants research vol. 27,1 (2016): 39-46. doi:10.1111/clr.12509.

87.Schroeder J. E., Mosheiff R. (2011). “Tissue engineering approaches for bone repair: Concepts and evidence”; International Journal of the care of the injured, 42(6), 609-613. https://doi.org/10.1016/j.injury.2011.03.029.

88. Zimmermann G., Moghaddam A. (2011). “Allograft bone matrix versus synthetic bone graft substitutes”; International Journal of the care of the injured, 42(2), 16-21. https://doi.org/10.1016/j.injury.2011.06.199.

89. Cicciù M. (2020). “Growth Factor Applied to Oral and Regenerative Surgery”; International Journal of Molecular Sciences. 21(20), 7752; https://doi.org/10.3390/ijms21207752.

90. Hussain I, Moharamzadeh K, Brook IM, et al. Evaluation of

osteoconductive and osteogenic potential of a dentin-base bone substitute using a calvarial de- fect model. Int J Dent 2012:2012:396316.

91. Jianan Li, Juan Yang, Xiaozhong Zhong, et al. Demineralized dentin matrix composite collagen material for bone tissue. J Biomater Sci Polym Ed 2013: 24(13):1519-28.

92 Kim YK, Bang KM, Murata M, et al. Rospective clinical study of allogenic demineralized dentin matrix for alveolar bone repair. J Hard Tissue Biol 2017;26:95-102.

93. Minamizato T, Koga T, Takashi I, et al. Clinical application of autogenous partially demineralized dentin matrix prepared immediately after extraction for alveolar bone regeneration in implant dentistry: a pilot study. Int J Oral Maxillofac Surg 2018;47:125-32.

94. Murata M, Akazawa T, Takahata M, et al. Bone induction of human tooth and bone crushed by newly devel- oped automatic mill. J Ceram Soc Jpn 2010;118(6):434-7

95. Kim YK, Yun PY, Um IW, et al. Alveo- lar ridge preservation of an extraction socket using autogenous tooth bone graft material for implant site devel- opment: prospective case series. J Adv Prosthodont 2014;6(6):521-7.

7 List of Figures and Tables