1. Scientific background

The TMJ and its associated structures (mandibular condyle, articular disc, glenoid fossa, and articular eminence) are at the center of mandibular function, motion, and distribution of loads produced by everyday tasks, such as chewing, swallowing, and speaking. Degeneration of the TMJ and TMJ disorders (TMDs) are associated with morphological and functional deformities of joint structures[1] which gradually lead to abnormalities of the articular disc and joint surfaces, with ultimate degeneration and dysfunction of the associated structures [2]. Thus, patients eventually are dealt with debilitating symptoms including painful joint sounds, restricted range of motion, and chronic orofacial pain. From an epidemiological standpoint, reports on TMJ disorders have shown that a considerable portion of the population is affected by these disorders, ranging from 16% to 59% for patients suffering from symptoms such as pain, clicking, limited motion and crepitus[3]. Moreover, degenerative in general are common in older patients, unusual age distribution exists for degenerative disorders of the TMJ ranging from 20 to 50 years of age [4,5].

Management of TMJ disorders range from non-invasive conservative management that includes soft diet, physiotherapy and occlusal splints, and up to joint surgery such as arthrocentesis, disc repositioning, disc removal or reshaping of the articular surfaces[6–9]. In the case of end-stage TMJ disorders, severe degenerative joint disease is not responsive to conservative therapy or surgical intervention within the joint, rendering the joint components as non-salvageable with minimal to no function at all[10]. Thus, removal of either the bony or cartilaginous component of the joint, or both, is indicated. Additional etiologies for end-stage disease state include irreparable joint structures secondary to trauma, developmental disease or TMJ pathologies[11]. In these cases, reconstruction of the TMJ is performed to restore function and alleviate symptoms[12–14]. Reconstruction of the TMJ has evolved in the past century, integrating several techniques. One option is to use harvested autografts, commonly used for younger patients. However, these are associated with major disadvantages such as limited autograft volume, tissue site morbidity, and substantial post-operative complications including resorption of the implants and necrosis [15–19]. As for alloplastic implants, various materials have been introduced with mixed results[20–23]. Despite their biocompatibility and mechanical strength, synthetic

joint replacements remain as a permanent foreign material within the body and only bond with native bone tissue through the mechanical interlocking with no bioactivity, which can result in loosening and wear of implants [24–26]. Thus, fabricating a biological component of the TMJ able to treat both intermediate and end stage disease is one of the most elusive challenges both biomedical engineers and surgeons face today.

Current preclinical studies have focused on producing either the articular disc or the mandibular condyle. Translational attempts for TMJ disc fabrication have focused on the use of collagen-based structures[27], stem cells[27–29] and polymers[30,31]**.** 3D printing of polymers was also employed for producing the articular disc with appropriate areas of convexity and concavity[31].

To this day, **no reports exist on the reproduction of a complete TMJ system, consisting of the mandibular condyle, glenoid fossa, articular disc and the corresponding ligaments that allow mandibular movement.** Rather, each component was fabricated as an isolated system. Moreover, **currently there is no protocol focused on reestablishing the cartilaginous components of the TMJ based on accurate patient-specific anatomy, retrieved from magnetic resonance imaging (MRI).** Thus, this proposal aims to combine appropriate cells, imaging, and 3D-baesd deposition of polymers and biological materials to create a holistic *in vitro* TMJ system, containing all components of the TMJ. **We will rely on previous work[32–34] and experience in fabricating anatomical scaffolds and the use of patient-derived mesenchymal stromal cells, to bioprint a TMJ device containing the articular disc and supporting cartilage and subchondral bone system**. We believe this work would translate into a clinical setting to aid patients in need of joint replacement.

2. Research objectives

Aim 1: Establishment of an in vitro TMJ device for the cultivation, structural evaluation and biological assessment of a system containing engineered articular surfaces, articular disc, and ligaments.

We rely on previous results and experience in cultivating cells and formulating a device with relevant compartments for each component, allowing movement of an articular disc between articular surface designed to mimic the mandibular condyle and glenoid fossa and eminence. Chambers will be prepared to allow the introduction of different cues and media. The system is prepared as to allow removal from chambers in one piece.

Aim 2: Cellular and biological induction of bony and articular components of the modeled TMJ.

Adipose derived MSCs will be used to enhance bone and articular components as previously described[33,34], supplemented with a novel hyaluronic acid based hydrogel used to enhance the matrix of elastic and cartilaginous components[35]. A k-carrageenan supplemented HA-hydrogel bone gel[36,37] will be used to promote osteogenesis of the sub-chondral bone components (condyle and fossa). Adipose derived endothelial cells (HAMECs) will be used according to previous experience and reports to promote vascular induction of bony compartments.

Aim 3: In vivo assessment of safety and function of the engineered TMJ system.

The engineered TMJ system will undergo implantation in a rat model, in which the TMJ system will be removed, and assessed *in vivo* and *ex vivo*. Function is followed regularly as animals return to normal function, and final assessment is performed 8 weeks after implantation.

3. Significance and innovation of the research program

The proposed project aims to produce, for the first time, a holistic joint system for the TMJ, *in vitro*, for longitudinal evaluation of development and maturation of the different components that reside within tמשגןhe joint. The 3D based design allows both the connectivity and compartmentalization of different parts that comprise the modeled joint. Further induction with appropriate mesenchymal and endothelial cells, with their proven clinical relevance, will enhance

the ECM deposition and maturation of the system. In addition, we will use a novel HA-based hydrogel recently shown to be injectable, biocompatible, able to undergo structural recovery and that can undergo tunning to support different diffusion rates of oxygen, nutrients, and waste depending on the desired tissue structure. k-carrageenan will complement these components by promoting osteoblast differentiation and bone organization in the modeled subchondral bone. Thus, the combination of these cells, biomaterials and factors will enable the *in vitro* maturation of a novel modeled TMJ, which will be able to undergo implantation and engraftment for regenerative purposes.

4. Research plan

4.1. Working hypothesis

The major hypothesis of this project is that combination the cumulative design of a TMJ system, comprising bone-supported articular surfaces and an articular disc, together with appropriate polymers and injectable biomaterials able to mimic the native cellular microenvironment, will enable the fabrication of a modeled joint tissue. We will reach this goal by designing a compartmentalized system able to simulate each component, able to undergo *in vitro* monitoring and evaluation, based on our experience with 3D-bioprinting and anatomical fabrication of polymeric scaffolds, use of tunable hydrogels and clinically relevant cells.

4.2. Design and methods

Aim 1: Establishment of an in vitro TMJ device for the cultivation, structural evaluation and biological assessment of a system containing engineered articular surfaces, articular disc, and ligaments.

The fabrication of the TMJ system will commence with 3D printing of the components of the joint, based on previous reports[32,38,39] and our own experience in modeling and fabricating polymeric scaffolds, by deposition of sacrificial polymers (BVOH). The design depicted in **Figure 1** enables the

introduction of both PDMS and PCL, to create a polymeric system entrapped within a PDMS chamber. The system is designed to enable movement of the joint structures under sterile conditions, mimicking the hinge and translational movement of the condyle relative to the fossa. High resolution micro-computed tomography and scanning electron microscopy will enable in-depth evaluation of the structure microarchitecture.

In the next phase, GFP-labeled human dermal fibroblasts (GFP-HNDFs) will be initially used to evaluate attachment and viability of cells introduced into the system within the first several days of culture. Next, the induction process depicted in **Figure 2** will include (I) the introduction of HA-hydrogel for articular components and k-carrageenan supplemented HA-hydrogel for subchondral bone, (II) the loading and expansion of MSCs within the joint components, (III) the introduction of different cultivation media according to the desired tissue formation, and (IV) loading of HAMECs into the subchondral bone compartment to induce vascularization *in vitro*.

Aim 2: Cellular and biological induction of bony and articular components of the modeled TMJ.

Adipose derived MSCs will be used as support cells to enhance deposition of ECM within bone or articular components as previously described[34,40]. The different compartments will be subjected to either osteogenic or chondrogenic media and supplemented with a novel hyaluronic acid-based hydrogel used to enhance the matrix of both articular disc and cartilage[35]. A k-carrageenan functionalized HA-hydrogel[37] is used to promote osteogenesis of the sub-chondral bone components (condyle and fossa). Adipose derived endothelial cells (HAMECs) will be used according to previous experience and reports to promote vascular induction of bony compartments.

Different compartments will undergo sampling of media to evaluate secretion of paracrine factors, ECM organization, as well as detachment of cells and biocompatibility. Cells viability is followed at least 24 and 72 hours after cellular loading. Immunohistochemistry is performed to follow ECM deposition (aggrecan, elastin, collagen2, collagen 1, bonesialoprotein2), cellular commitment and organization (runx2, a-smooth muscle actine, CD31) as well as histological stains to evaluate tissue organization (h&e, alcian blue, safranin O). MicroCT scanning of samples stained with iodine will

allow 3D-visualization of the deposited ECM within each structure as previously reported[41]. Moreover, the spatial organization of ECM components will be evaluated within the microenvironment based on eigenvector analysis[42]. These conventional assays will verify the induction of our compartmentalized TMJ structure and provide the necessary feedback for tuning the biomaterials and factors used for each structure of the modeled joint.

Aim 3: In vivo assessment of safety and function of the engineered TMJ system.

We will evaluate the engraftment, survival and function of the modeled TMJ in a rat model. Rats will undergo removal of the TMJ disc, the articular surfaces from the glenoid fossa and the condyle according to previous reports on TMJ surgery in murine models[43,44]. Our TMJ system with its engineered articular surfaces, disc and subchondral bone will be attached to reinstitute the joint. **Joint function** in the form of dietary consumption (soft and hard), body weight and maximal mouth opening will be monitored constantly throughout the duration of the experiment. Eight weeks after surgery, **CT and MRI imaging** will be employed to evaluate the integration and survival of both bony structures and cartilaginous components. Two and eight weeks after implantation, **histology, immunohistochemistry and morphometric analysis** are performed on explanted TMJs to assess their integration, inflammatory response and survival. Staining will include h&e and alcian blue for general joint tissue morphology and ECM deposition, vascular staining such as CD31, alpha smooth muscle actin, and laminin, ECM staining including Collagen2 and Collagen1, and inflammatory staining of pan macrophages marker CD68 as well as M1 and M2 cells within the engraftment site. Human nucleic acid stain will enable the evaluation of cellular survival after implantation.

The proposed treatment will allow us to evaluate the safety and function of the modeled joint. We will follow rehabilitation of animal subjects while adhering to all care and safety regulations, making sure animals are able to feed and survive with minimal suffering or stress.

4.3. Preliminary results

4.3.1. Design, 3D-printing and fabrication of the *in vitro* TMJ system

Our team specializes in 3D-based design and fabrication of constructs. This methodology has been employed for neural, cardiac, and auricular tissue engineering. The fabrication process is outlined in **Figure 3**, and includes design of the TMJ system with corresponding areas for different tissue components, 3D printing of a sacrificial mold by using a water-soluble fugitive polymer (BVOH), introduction of the polymer and lyophilization of the construct.

4.3.2. Fabrication, cellular loading and vascularization of an injectable HA-hydrogel for tissue engineering

We have recently shown that a novel hyaluronic acid-based hydrogel can be used as a substrate for tissue engineering purposes. The hydrogel is highly compatible, can undergo deformation and recovery under stress, and support cellular proliferation while stability of the gel was verified *in vivo* after injection. Moreover, we were able to support vessel network formation within the hydrogel by loading co-cultures of MSCs and endothelial cells (**Figure 4**).

4.3.3. Using k-carrageenan as a supplement for inducing osteogenic differentiation

k-carrageenan, a natural polysaccharide extracted from red seaweeds, was proven to increase pre-osteoblasts adhesion, proliferation, osteogenic differentiation and metabolic activity, rendering it a potent inductive factor for bone regeneration[36] (**Figure 5**). When k-carrageenan was used to functionalize coating of titanium implants, acceleration of osteogenic matrix deposition and mineralization was verified, with upregulation of corresponding genes[37] (**Figure 6**). Thus, we intend to harness this agent to promote the formation of subchondral bone in our TMJ model.

4.3.4. An *in vivo* system to evaluate maxillofacial defects reconstruction and engraftment

The experience we gained in maxillofacial tissue models will enable the implementation of the designed TMJ system into an animal model. The surgical approach utilized will be based on a model already used of maxillofacial defect reconstruction of the zygomatic arch in a

murine model. Both microCT imaging and engraftment of blood vessels were assessed in such a model, with great success (**Figure 7**).

4.4. Facilities and infrastructure

Prof. Qingbin's lab

Prof. Srouji's lab

Our lab, stretching over **** squared meter, is a part of the Galilee medical center research institute (GMCRI, https://www.gmcri.org.il), a core facility in charge of all basic, clinical and translational research activities at the Galilee medical center. The GMCRI is also affiliated with the Bar-Ilan university Azrieli faculty of medicine. Thus, we have full access to both clinical infrastructure and advanced imaging, material science and animal facilities on site and in the faculty. Our lab, consisting of offices and joint working spaces, possesses a tissue culture room, molecular stations, material science station, two 3D printing laboratories, and a dedicated digital 3D design room equipped with haptic feedback station. Major items available for our laboratory use include several biological cabinets, a stereolithography 3D printer (Fabpro 1000), polymer 3D printer (Prusa MKi3), a state-of-the-art biological 3D printer (BioX 2.0), lyophilizer, rheometer, evaporator, paraffin histology station, cryostat, incubators, cell counter, inverted fluorescent microscope, refrigerators and freezers and all necessary support infrastructure for tissue culture. We also hold a one-site animal facility for exclusive use of the GMCRI, under supervision of the Bar-Ilan university. Shared facilities include confocal microscopy, Incucyte, STED, microsCT, MRI, SEM, TEM, EMPT, high speed slide scanners, high-speed ultra-centrifuge, clean chambers, FASC, QPCR and bioluminescence imaging.

Our lab is currently staffed by a P.hD-level research associate, M.Sc-level lab engineer, and 6 graduate students (P.hD and M.Sc).

The joint infrastructure and resources together with the accumulated experience and devotion of both labs, will be able to successfully reach the goals and aims set in this proposal.

4.5. **expected results, pitfalls and alternatives.**

In the proposed study we intend to fabricate the modeled joint device, able to undergo both specialized tissue induction and monitoring in vitro. The device should support compartmentalized differentiation and growth of cells and biological matrix of different function (bone and cartilage), should be biocompatible with minimal cytotoxicity. We expect to create within several weeks of culture a composite device, containing subchondral and chondral components with a specialized articular disc apparatus. We expect cells to differentiate to ether chondrogenic or osteogenic lineage, depending on the desired compartment. Once the modeled TMJ device has undergone sufficient maturation, a rat TMJ defect study will commence, expected to demonstrate rehabilitation of jaw movement and function. We believe that as the implanted devices become more mature and vascularized in vitro, their integration and survival in vivo will be verified.

Pitfalls and alternatives

One major challenge is the simultaneous cultivation of different components within one device. In our design, the PDMS chamber is planned to support differential cultivation of both bone and chondral tissue. In case satisfactory separation of compartments is not achieved, pores membranes (0.2-0.8 µm) will be introduced into the PDMS mold to aid in facilitating separation of compartments. Additional step may include differential dynamic culture in which different perfusion rates of media are introduced to enhance differential growth of compartments, as previously reported[45].

Another challenge may reside in the fact that the physical microarchitecture does not enhance the development of bone vs cartilage. In that case, the polymer density in each chamber can be further tuned, to change the porosity and density of deposited PCL polymer.

The development of a vascular system within the modeled bone is a crucial step in enhancing engraftment after implantation. However, the transition of MSCs as support cells toward an osteoblastic lineage may degrade or inhibit development of vascular networks. Moreover, *in vitro* vessel networks may reach their prime before the bone compartment has matured, rendering them unfavorable for implantation. Thus, additional option would be to create vascular organoids separately using SLA based PDMS chips, which will be loaded onto the bone compartment in the device toward the end of the cultivation period.

An additional challenge stems from the use of a highly inductive osteogenic agent – the k-carrageenan within the bone compartment. The success of the differential cultivation is based on adequate adherence of the agent to the HA-hydrogel injected into the bone compartment. Unsupervised release of the k-carrageenan into the system may hamper differentiation of MSCs in cartilaginous compartments. Thus, we plan to devote resources to tune the incorporation of k-carrageenan into the HA-hydrogel. In case this cannot be achieved, a simplification of the system will include the differentiation of MSCs into osteoblasts and osteocytes prior to loading them into the bone compartment.

5. **Figures**

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