Osteosarcoma (OS) is the most common bone tumor in pediatrics. After resection, allografts or metal endoprostheses reconstruct bone voids, and systemic chemotherapy is used to prevent recurrence. This urges the development of novel treatment options for the regeneration of bone after excision. We utilized a previously developed biomimetic, biodegradable magnesium-doped hydroxyapatite/type I collagen composite material (MHA/Coll) to promote bone regeneration in the presence of chemotherapy. We also performed experiments to determine if human mesenchymal stem cells (hMSCs) seeded on MHA/Coll scaffold migrate less toward OS cells, suggesting that hMSCs will not contribute to tumor growth and therefore the potential of oncologic safety in vitro. Also, hMSCs seeded on MHA/Coll had increased expression of osteogenic genes (BGLAP, SPP1, ALP) compared to hMSCs in the 2D condition, even when exposed to chemotherapeutics. This is the first study to demonstrate that a highly osteogenic scaffold can potentially be oncologically safe because hMSCs on MHA/Coll tend to differentiate and lose the ability to migrate toward tumor cells. Therefore, hMSCs on MHA/Coll could potentially be utilized for bone regeneration after OS excision.

Keywords
Osteosarcoma, biomimetic, MHA/Coll scaffold, osteogenesis, chemotherapy, bone regeneration

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for the bone to repair. During normal endochondral bone development and direct bone formation, MSCs differentiate into osteoblasts to create new bone. In OS, osteoprogenitor cells can acquire mutations that cause uncontrolled cell growth. In addition, when the time period of osteogenesis is prolonged, osteoprogenitor cells have an increased risk to become OS cells. Therefore, MSCs committed to the osteogenic lineage can become the cell of origin for OS. Moreover, MSCs can directly influence OS cells’ behavior and growth, rendering them crucial players in pathways that control the shift toward regeneration rather than tumor recurrence. Once MSCs have reached the tumor, the cross talk between MSCs and OS tumor cells favors angiogenesis and the formation of new vessels to support tumor growth. In addition, MSCs secrete cytokines and soluble growth factors that aid in the migration, proliferation, “stemness,” metabolic reprogramming of tumor cells, and immune escape.

We previously developed a biomimetic magnesium-doped hydroxyapatite/type I collagen-based material (MHA/Coll) synthesized through a biologically inspired method, which recapitulates the bone mineralization process. The material resembles the main components of the bone’s extracellular matrix such as type I collagen and hydroxyapatite. The hydroxyapatite is doped with magnesium, an ion naturally present within the natural apatite structure of young bone, because studies have demonstrated that magnesium deficiency affects all stages of skeletal metabolism. In vitro and in vivo, we have previously demonstrated that MHA/Coll is able to promote accelerated osteogenesis promoting bone formation in ectopic and orthotopic sites. However, our material has only been used to repair critical size defects of bone. We have yet to explore the ability of MHA/Coll to repair bone defects following resection of primary tumors, such as OS.

The ability to repair bone voids after tumor resection is a difficult task because of the complexity of the cycle and cross talk of MSCs, pre-osteoblasts, osteoblasts, and tumor cells. This is evident by many groups being hesitant to regenerate bone in an area that was previously occupied by a tumor because the MSCs that occupy the scaffold could potentially contribute to the rapid proliferation of residual OS cells. However, we hypothesize that the osteogenic property of MHA/Coll will more quickly induce MSCs toward osteoblast terminal differentiation, rather than OS development and spread. MHA/Coll can be manufactured as a spongy scaffold or a thin membrane without losing its functional properties (i.e. structure, biochemical composition, and physical properties) depending on the surgical need. The MHA/Coll scaffold is used in vitro to mimic the in vivo bone environment, while the MHA/Coll membrane can be used in vivo as a surgical tool when wrapped around metal hardware to facilitate bone regeneration and integration. Because the MHA/Coll accelerates differentiation of MSCs faster than in the 2D environment, we hypothesize that OS development from recruited MSCs is less likely.

In addition, the current treatment for OS includes systemic chemotherapy such as doxorubicin, methotrexate, and cisplatin. Multiple studies that have demonstrated that MSCs have resistance to multiple chemotherapeutics, suggesting that the differentiation of MSCs into osteoblasts and therefore the osteogenesis will be unaffected by the presence of chemotherapy in vitro.

Aims

This study will be the first to examine in an in vitro 3D setting if—osteogenesiscan be affected by the chemotherapeutic treatments. We have two aims: (1) to determine if hMSCs on MHA/Coll are able to undergo osteogenesis even in the presence of chemotherapeutics in vitro, and (2) to determine if hMSCs on MHA/Coll cause hMSCs to migrate less toward patient-derived OS cells or OST conditioned media. We hypothesize that a highly osteogenic material will be oncologically safe because MSCs recruited to MHA/Coll will be driven toward osteoblasts and therefore be able to promote osteogenesis in the presence of chemotherapy.

Results

MHA/Coll membrane and scaffold, bone chip characterization

The morphology of the MHA/Coll scaffold (Figure 1(a)), MHA/Coll membrane (Figure 1(b)), and bone chip (Figure 1(c)) were characterized by scanning electron microscopy (SEM) (Figure 1(a)–(c)). As expected, the porous MHA/Coll scaffold is characterized by interconnected porosity with an average pore size of 2000 μm and an overall porosity of 80% (Supplemental Figure 1C). At lower magnification, SEM images showed the different topography between MHA/Coll scaffold and membrane. MHA/Coll membranes have been fabricated by solvent casting method, which not allow for porosity formation. At higher magnifications, the mineralization of the collagen fibers was observed in both MHA/Coll scaffolds and membranes (Figure 1(a)). To determine if the morphology of the MHA/Coll scaffold were similar to human trabecular bone, SEM of bone chip was compared. Similar pore size was observed.

Mechanical properties of both the MHA/Coll scaffold and bone chip were assessed with compression tests and the results are shown in Supplemental Figure 1. Young’s modulus of MHA/Coll scaffold and bone chip, evaluated in their respective linear regions, resulted in 791.6 ± 25.4 kPa for the bone chip and 47.14 ± 2.5 kPa for the MHA/Coll scaffold.
The XRD of MHA/Coll was previously published by our group. Briefly, compared to commercially available stoichiometric hydroxyapatite, our MHA/Coll was characterized by low crystallinity, and a similar pattern to that of the mineral phase of human bone. Also, when MHA/Coll was nucleated, it displayed a pattern typical of amorphous phases.

We then further performed TGA analysis on MHA/Coll membrane, MHA/Coll scaffold, and bone chip to determine the amount of the mineral phase that was nucleated on the type I collagen. Figure 1(d) demonstrates that the mineral content of the MHA/Coll membrane, MHA/Coll scaffold was 56 wt%, which is comparable to human trabecular bone (bone chip, 53 wt%). In addition, MHA/Coll membrane and MHA/Coll scaffold were equivalent.

The FTIR spectra (Figure 1(e)) showed the characteristic collagen peaks at Amide I (1700–1600 cm⁻¹) and amide II (1600–1500 cm⁻¹), related to the stretching vibration of C=O bonds and to C–N stretching and N–H bending.
vibration respectively. The sample contained C=O, C–N, and N–H bonds. The Amide III region (approximately 1200–1300 cm\(^{-1}\)) is related to the C–N and C–C stretching, N–H bonds, and CH\(_2\) wagging from the glycine backbone and proline side chain. In addition, the peak at 900–1000 cm\(^{-1}\) demonstrates that the collagen was mineralized. The same peaks were observed in both MHA/Coll scaffold, MHA/Coll membrane, and naïve human trabecular bone (bone chip).

**hMSCs viability on MHA/Coll membrane and MHA/Coll scaffold**

The viability of hMSCs seeded on the MHA/Coll membrane and scaffold was assessed using flow cytometry compared to hMSCs in the 2D condition, 48 h after seeding. No differences in hMSCs viability were found between the three conditions (Supplemental Figure 2).

**hMSCs loss of surface markers in 2 versus 3D**

hMSCs seeded on the MHA/Coll scaffold or in the 2D condition were placed in aMEM media or osteogenic media. MHA/Coll Scaffold was utilized to mimic the in vivo 3D environment in an in vitro setting. At 7, 14, and 21 days, cells were collected and the presence of hMSC surface markers (CD90, CD105, CD73) was evaluated to determine how MHA/Coll affected the differentiation. As early as 7 days, almost all hMSCs seeded on MHA/Coll in either aMEM or osteogenic media lost their surface markers (aMEM media: CD90 0.02%, CD105 0.66%, CD73 0.49%, osteogenic media: CD90 0.02%, CD105 0.79%, CD73 0.25%) (Figure 2(a)–(c)). Figure 2(d) show the % of hMSCs triple positive for CD90, CD105, and CD73 (97.63% and 6.97% in 2D aMEM and OS media respectively, while 0% in 3D condition aMEM and OS). This demonstrated the loss of all these surface cell markers occurred earlier on MHA/Coll compared to hMSCs in the 2D condition in osteogenic media. Although the presence or loss of stem cells markers is not predictive of the osteogenesis potential, our previous and present results may suggest that MHA/Coll could accelerates the differentiation of hMSCs toward osteogenic lineage. To support this hypothesis, in our previous work we found, in vivo, osteogenic commitment as early as 7 days.\(^{32}\)

**Interaction between hMSC and PDX-derived osteosarcoma cells on MHA/Coll scaffold and MHA/Coll membrane**

To evaluate if hMSCs recruited to MHA/Coll in the in vivo setting would accelerate the growth of residual OS cells following tumor resection, the migration of hMSCs toward human derived OS cell lines (TCCC-OS94 and TCCC-OS202) or PDX-derived conditional media (CM) was evaluated in the 2D and 3D conditions (Figure 3(a)). Significantly less migration of hMSCs toward primary or metastatic human-derived OS cell lines was observed when hMSCs were seeded on the MHA/Coll scaffold or membrane (p-value < 0.001, Figure 3(b)). Similarly, hMSCs seeded on the MHA/Coll scaffold were significantly less likely to migrate toward PDX-derived CM (p-value < 0.0001) (Figure 3(c)). Our in vitro system suggests that hMSCs seeded on MHA/Coll are less likely to migrate toward OS cells diminishing their contribution to tumor growth by differentiating in osteoclast; however, we cannot exclude a potential paracrine effect.

This observation was supported by the fact that when cells were detached after 48 h from the seeding and subsequently seeded on the transwell insert, we again observed significantly less migration toward PDX-derived CM (p-value < 0.0001) or PDX OS cells (p-value < 0.0001) of hMSCs that were seeded on the MHA/Coll scaffold or MHA/Coll membrane (Supplemental Figure 3). This suggests that hMSCs in 3D condition undergo a change after already 48 h and starting to probably differentiate in osteoblast and therefore they are less likely able to migrate toward OS cells compared to hMSCs that were not on MHA/Coll.

**IC-50 of PDX-derived osteosarcoma cell lines against chemotherapeutics**

The IC-50 of two different human PDX-derived OS cell lines (TCCC-OS94 and TCCC-OS202) was evaluated against cisplatin, doxorubicin, and methotrexate (Table 1). For cisplatin, the IC-50 was found to be 2.28 and 6 μM for TCCC-OS94 and TCCC-OS202 respectively (Supplemental Figure 4A and B). For doxorubicin, IC-50 was found to be 0.25 and 0.13 μM for TCCC-OS94 and TCCC-OS202 respectively (Supplemental Figure 4C and D). We were not able to determine methotrexate IC-50 (Supplemental Figure 4E and F) therefore all the experiments will only include doxorubicin and cisplatin.

**Viability of hMSCs when exposed to chemotherapeutics**

To determine the viability of hMSCs when exposed to cisplatin and doxorubicin, cells were plated in the 2D condition and exposed to one of the two chemotherapeutics at the IC-50 dose in aMEM or osteogenic media. Cells were collected at 7 or 21 days, and viability was evaluated using MTT. We did not observe a difference in hMSCs viability in aMEM condition with or without chemotherapeutics and hMSCs cultured in osteogenic media showed even higher viability compared to cells in aMEM media (Figure 4). This suggests that the viability of hMSCs are not affected by chemotherapeutics. However, we needed to
Figure 2. hMSC markers at 7, 14, and 21 days in 2D versus 3D environment: quantification graphs and percent of surface expression markers of hMSCs seeded in the 2D condition or in 3D (MHA/Coll scaffold) in aMEM media or osteogenic (OS) media at 7, 14, and 21 days: (a) CD90, (b) CD105, (c) CD73, (d) hMSCs (triple positive for CD90, CD105, and CD73). About 7% of hMSCs in the 2D condition in osteogenic media lost their surface markers at 7 days, while 100% of hMSCs lost their markers when seeded on MHA/Coll scaffold. OS: osteogenic media. Statistical analysis by Pearson correlation analysis and compared to the aMEM 2D condition. All conditions were statistically significantly different compared to aMEM 2D at all time points (T7, T14, T21) (p value <0.001).
determine if properties (other than viability) are affected by chemotherapy in the in vitro setting.

**Osteogenesis of hMSCs on MHA/Coll membrane**

To determine if MHA/Coll resulted in accelerated osteogenesis or if chemotherapeutics affected osteogenesis, RNA was isolated at 7 and 21 days after culture and the expression of three genes (BGLAP, SPP1, ALP) was evaluated (Figures 5 and 6). In detail, hMSCs undergoing osteogenesis in the 2D condition had increased expression of BGLAP at 7 days even when exposed to doxorubicin (Figure 5(a) and (c), \( p \)-value < 0.0001 for TCCC-OS202, \( p \)-value < 0.05 for TCCC-OS94) or cisplatin (Figure 6(a)).

**Table 1. Summary of IC50 PDX-derived OS cell lines.**

| hPDX cell lines | IC50 Cis (\( \mu \)M) | IC50 Dox (\( \mu \)M) |
|-----------------|----------------------|----------------------|
| TCCC-OS94      | 2.28                 | 0.25                 |
| TCCC-OS202     | 6                    | 0.13                 |

Figure 3. Migration of hMSC decreased by MHA/Coll: (a) schematic representation of experimental set-up of hMSCs migration towards PDX-derived OS cells (TCCC-OS94, TCCC-OS202), (b) graph quantification of hMSCs migration towards OS PDX cell lines in αMEM or PDX media, (c) representative image of hMSCs that migrated from the top to bottom of the transwell insert, (d) schematic representation of experimental set-up of hMSCs migration towards conditional PDX-derived OS media, (e) graph quantification of hMSCs migration towards PDX-derived OS conditional media (CM), (f) representative image of hMSCs that migrated from the top to bottom of the transwell insert. hMSCs seeded on MHA/Coll scaffold or membrane demonstrated significantly less migration towards OS PDX cells. Human PDX-derived OS cell lines: TCCC-OS94, TCCC-OS202; OS: osteogenic media; CM TCCC-OS94 and CM TCCC-OS202: conditional media derived from respective PDX-derived OS cell lines. ***\( p \)-value < 0.001, scale bar = 500 µm.
and (c), p-value < 0.01 for TCCC-OS202, p-value < 0.0001 for TCCC-OS94), as well as for SPP1 at 7 days when exposed to doxorubicin (Figure 5(a) and (c), p-value < 0.0001 for TCCC-OS202 and TCCC-OS94) or cisplatin (Figure 6(a) and (c), p-value < 0.0001 for TCCC-OS202 and TCCC-OS94). In the 3D condition, the MHA/Coll membrane was utilized because the mineralization of the MHA/Coll scaffold interfered with the ability to isolate the quantity and quality of RNA for gene expression. When hMSCs were seeded on the MHA/Coll membrane, increased expression of genes involved in osteogenesis were also observed. BGLAP was expressed at higher levels as early as 7 days when hMSCs were in aMEM media or osteogenic media exposed or not to doxorubicin (Figure 5(b) and (d), p-value < 0.0001 for the IC-50 to TCCC-OS202, p-value < 0.001 for the IC-50 to TCCC-OS94) or cisplatin (Figure 6(b) and (d), p-value < 0.0001 for the IC-50 to TCCC-OS202 and the IC-50 to TCCC-OS94). Increased expression was also observed for ALP when hMSCs were exposed or not to doxorubicin (Figure 5(b) and (d), p-value < 0.0001 for the IC-50 to TCCC-OS202 and the IC-50 to TCCC-OS94) or cisplatin (Figure 6(d), p-value < 0.05 for the IC-50 to TCCC-OS94).

To further characterize osteogenesis differentiation, von Kossa staining is usually used to evaluate calcium deposition. However, due to the mineralization of MHA/Coll, significant calcium deposition was observed on the scaffold and membrane even in absence of cells (Supplemental Figure 5). Therefore, the ions within MHA/Coll interfere with the von Kossa staining and this protocol cannot be applicable for our specific in vitro system.

**Interaction between hMSC and TCCC-OS cells with chemotherapeutics**

To evaluate if the migration of hMSCs toward TCCC-OS cells or PDX-derived CM was affected by the presence of cisplatin or doxorubicin, a migration assay was performed. hMSCs were seeded in the 2D conditional and exposed to the IC-50 of the respective chemotherapy in either osteogenic or aMEM media for 21 days (Figure 7). There was significantly less migration of hMSCs in osteogenic media independently from the presence of the chemotherapeutics toward both TCCC-OS cell lines (Figure 7(b), p-value < 0.0001) and PDX-derived CM (Figure 7(a), p-value < 0.0001). This further suggests that hMSCs recruited to MHA/Coll in vitro will not migrate toward the tumor and this is not more likely to be affect if hMSCs exposed to chemotherapeutics.

**MSC differentiation in 2 versus 3D on MHA/Coll membrane with chemotherapy**

To further evaluate if hMSCs on MHA/Coll underwent osteogenesis even in the presence of chemotherapy, the loss of MSC surface markers was evaluated by flow
cytometry. MHA/Coll membrane was used for the 3D condition, because at 21 days, hMSCs seeded on the MHA/Coll scaffold are difficult to detach and isolate to perform flow cytometry experiments. hMSCs were exposed to chemotherapeutics for the entire 21 days. When hMSCs were exposed to either doxorubicin or cisplatin, they lose the surface markers at 21 days in the 2D condition in aMEM and Ost media (Figure 8(a) and (b)). This effect was also observed in hMSCs seeded on MHA/Coll membrane at 21 days in presence of chemotherapeutics in Ost media (Figure 8(b)) and aMEM media (Supplemental Figure 6A and B). This confirms that both cisplatin and doxorubicin do not have any effects on the osteogenesis differentiation of hMSCs.

**Discussion**

Current treatment for OS involves both chemotherapy and surgery, limb salvage with reconstruction of weight-bearing bones (utilizing endoprosthesis or biological replacement) or limb amputation.  Current treatment greatly involves the use of allografts or autografts. Amputation is utilized when the tumor involves invasion into the surround soft tissue or neuromuscular structures.  Treatment also involves the use of neoadjuvant and adjuvant chemotherapy. Chemotherapeutics most commonly administered include methotrexate, doxorubicin, and cisplatin. However, current treatment greatly involves the use of allografts or autografts. Amputation is utilized when the tumor involves invasion into the surround soft tissue or neuromuscular structures.  Treatment also involves the use of neoadjuvant and adjuvant chemotherapy. Chemotherapeutics most commonly administered include methotrexate, doxorubicin, and cisplatin. However, current treatment greatly involves the use of allografts or autografts. Amputation is utilized when the tumor involves invasion into the surround soft tissue or neuromuscular structures.  Treatment also involves the use of neoadjuvant and adjuvant chemotherapy. Chemotherapeutics most commonly administered include methotrexate, doxorubicin, and cisplatin.
decreases the quality of life of patients affected by OS. Although prosthetic materials have been utilized in the clinic for years, they cause incomplete healing, extended time to heal, prolonged non-weight bearing periods, increased fracture risk, infections, degenerative arthritis, and joint instability.40,42,54–60 The durability and lifespan of implants are critical features when dealing with pediatric patients affected by OS, who have not achieved their adult height; 40%–80% of prosthetic implants last <10 years, therefore requiring implant replacement within this adolescent population as they grow.6,7,9,61

To determine if bone regeneration can occur following OS tumor resection, it is first necessary to determine if the use of a scaffold to regenerate bone to repair bone defects following tumor resection would be oncologically safe. MSCs recruited to the scaffold could potentially increase the proliferation of OS cells because skip lesions and residual OS cells are common even if negative surgical margins are present. Though there have been numerous scaffolds utilized in bone regeneration, studies to evaluate the utility of bone regeneration following OS resection are limited. This is largely due to studies demonstrating a bidirectional communication between tumor cells and MSCs.28,62–65 Once MSCs have reached the tumor, the cross talk between MSCs and OS tumor cells favors angiogenesis and the formation of new vessels to support tumor growth. In addition, MSCs secrete cytokines and soluble growth factors that aid in the migration, proliferation, “stemness,” metabolic reprogramming of tumor cells, and immune escape.15,18,29,30,62

First, we demonstrated that hMSCs seeded on the MHA/Coll membrane and MHA/Coll scaffold had viability that

![Figure 6. OS media induces differentiation and MHA/Coll enhances differentiation at 7 days with cisplatin: (a) comparison of BGLAP, SPP1, and ALP expression in 2D aMEM and 2D osteogenic TCCC-OS202 culture with cisplatin, (b) comparison of BGLAP, SPP1, and ALP expression in osteogenic 2D and 3D TCCC-OS202 culture with cisplatin, (c) comparison of BGLAP, SPP1, and ALP expression in 2D aMEM and 2D osteogenic TCCC-OS94 culture with cisplatin, (d) comparison of BGLAP, SPP1, and ALP expression in 2D and 3D osteogenic media TCCC-OS94 with cisplatin. Values are depicted as mean ± SD. All groups were compared to the control of aMEM 2D in (a), (c), (e), and (g) and to the control of OS 2D in (b), (d), (f), and (h). Statistical analysis by unpaired t-test with Welch’s correction; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. aMEM: alpha minimum essential medium; OS: osteosarcoma; human PDX-derived OS cell lines: TCCC-OS94, TCCC-OS202; OS: osteogenic media.](image)
is equivalent to hMSCs in the 2D condition (Supplemental Figure 2) as previously reported (>90% at 7 days). Moreover, MHA/Coll showed the lowest rate of cell growth with respect to the 2D condition (less than 40% reduction at 1 week), suggesting hMSCs were differentiating instead of proliferating.
We subsequently exhibited that hMSCs seeded on MHA/Coll have decreased migration toward OS cells (Figure 3(b) and (e), Supplemental Figure 3), which is maintained even when hMSCs are exposed to chemotherapeutics (Figure 7(a) and (b)). This suggests that in the presence of MHA/Coll induces less crosstalk between hMSCs and OS cells, making hMSCs less likely to promote or accelerate OS cell growth. There have been studies that demonstrated cytokines secreted by OS cells can inhibit the MSCs osteogenic differentiation, causing MSCs to become more “pro-tumor.” However, one study conducted by Avril et al. tested if MSCs or osteoprogenitor cells had an effect on tumor growth. In the study it was found that while MSCs increased tumor size, the pre-osteoblasts did not. Moreover, it has been demonstrated that the longer MSCs are in an osteoprogenitor state, the more likely OS will develop. These results suggested that if the MSCs are quickly driven to complete the process of osteogenesis, the effect of MSCs on OS cells is limited. Our lab has previously shown that the MHA/Coll is not only osteoconductive, but also osteoinductive. MHA/Coll is able to induce osteogenic gene expression such as BGLAP, ALP, and SPP1 at higher levels compared to MSC cultured in osteogenic media in the 2D condition. We were able to provide additional support that hMSCs seeded on MHA/Coll undergo osteogenesis, even when exposed to chemotherapy (Figures 5(b), (d), 6(b), and

Figure 8. Flow cytometry of hMSCs stem cell markers expressed when exposed to IC50 of PDX-derived TCCC-OS94 and TCCC-OS202 at 21 days: (a) hMSCs exposed to amEM or osteogenic media ± IC50 of human PDX-derived osteosarcoma cell lines (TCCC-OS94, TCCC-OS202) at T21 days, (b) hMSCs on MHA/Coll membrane exposed to media ± IC50 of human PDX-derived osteosarcoma cell lines (TCCC-OS94, TCCC-OS202) at T21 days. Human PDX-derived OS cell lines: TCCC-OS94, TCCC-OS202. aMEM: alpha minimum essential medium; OS: osteogenic media.
Therefore, we believe that MHA/Coll not only pushes hMSCs to quickly differentiate into terminal osteoblasts, but also decrease the raw number of hMSCs that can migrate toward OS cells and therefore decreasing the number of hMSCs that could potentially crosstalk with OS cells. Both of these results suggest that MHA/Coll is oncologically safe and that potentially if MHA/Coll were implanted in vivo following OS resection, endogenous hMSCs recruited to MHA/Coll would not increase OS cell proliferation. However, additional experiments are needed in order to determine if there is a difference in the effect of the crosstalk between OS cells and undifferentiated hMSCs versus differentiated hMSCs. Our results would be further supported if it was determined that differentiated hMSCs exhibit less crosstalk with OS cells compared to undifferentiated ones.

We also demonstrated increased osteogenic genes expression at 7 days even when hMSCs were exposed to chemotherapeutics. In addition, hMSCs in the 3D osteogenic media condition had increased osteogenic gene expression compared to hMSCs in the 2D osteogenic media condition. We did not expect to observed equivalent gene expression between these two conditions; since both osteogenic media and MHA/Coll were able to induce osteogenic differentiation, hMSCs exposed to osteogenic media while cultured on MHA/Coll should have demonstrated a higher gene expression due to the synergistic effect.

Interestingly, expression of BGLAP and SPP1 in hMSCs exposed to doxorubicin or cisplatin in the 2D condition (Figures 5(a), (c), 6(a), and (c)) is increased compared to hMSCs in the 2D osteogenic media condition. Though there is an increase in gene expression, it is significantly less compared to the 3D condition. We hypothesize that the increased gene expression in the presence of drugs in our conditions is due to the large variability of expression levels observed among the different hMSCs cell lines used in these sets of experiments. In addition, as the hMSCs are isolated from different patients, there is variability in a multitude of factors such as different % of MSCs isolated, expression of osteogenic markers, the rate of proliferation and differentiation, and the differentiation ability.

Though we were able to demonstrate that hMSCs ability to undergo osteogenic differentiation and hMSCs viability is unaffected by chemotherapeutics, further experiments are needed to determine if hMSCs are unaffected by chemotherapeutics in other properties. We did not assess if chemotherapeutics affect the metabolism or the secretory properties of hMSCs. This would be important to determine in in vivo settings due to the complexity of downstream effects of metabolism and cytokines.

Our results are further supported by a recently published study examining the utility of using a polycaprolactone (PCL)/B-tricalcium phosphate (B-TCP) scaffold to regenerate bone in the canine following the resection of a distal OS tumor. Though the canine had local and metastatic recurrence 8-week post-operatively, neither neoadjuvant nor adjuvant chemotherapy was given. Moreover, CT scans demonstrated that bone regeneration occurred 6 weeks postoperatively. This suggests that scaffolds do push the MSCs recruited toward osteoblasts and bone regeneration. Additionally, because chemotherapy is the standard of care for OS treatment, even if metastases are not present prior to surgical resection, it is important to evaluate if chemotherapeutics do not affect hMSCs but also osteogenesis. Previous studies have demonstrated that MSCs secrete cytokines such as SDF-1 that can cause the chemotherapeutics resistance. This occurs via the promotion of MDR-1 (multi-drug resistance gene 1) expression as well as MRP (multidrug resistance-associate protein), drug efflux pumps. Our study also confirmed that the viability and osteogenesis of hMSCs are not affected by chemotherapy.

The clinical implications of this study could potentially provide a novel treatment option to repair bone voids following OS tumor excision. During surgical excision of the OS tumor, the surrounding healthy tissue where hMSCs reside may be affected due to the use of a laser or burr to burn the healthy tissue surrounding the tumor. However, there have been a multitude of studies that have demonstrated that hMSCs are able to migrate from both surrounding bone and skeletal muscle. In addition, our previous work has demonstrated that following a critical size defect, MSCs are still able to migrate to the MHA/Coll scaffold and to induce bone formation in a multitude of animal models (calvarial defects in rats, spinal defects in rabbits, and long bone defects in sheep). Moreover, in a critical size defect surgical model longitudinal cuts are made utilizing a burr, which stimulates the clinical surgical method. Therefore, we believe that the tools and methods utilized in the surgical excision of OS will not affect the ability of host hMSCs to migrate to MHA/Coll.

Though many groups have evaluated the utility of a scaffold preloaded with autologous cells to repair bone voids, our scaffold is unique since does not require the pre-loading of MSCs. We have previously demonstrated that MHA/Coll can be implanted and endogenous MSCs are subsequently recruited to the scaffold and undergo osteogenesis due to the mechanical, physical, and biochemical features of MHA/Coll. The ability to utilize a biomimetic scaffold that does not rely on the release of growth factors or stimulating bioactive that could promote tumor recurrence. Our MHA/Coll scaffold is unique because relies on self-instructing materials used as building blocks that inherently promotes stem cell recruitment, proliferation, and spontaneous differentiation toward the osteogenic lineage. Though endogenous hMSCs could potentially be affected by the presence of adjuvant chemotherapy or surgical excision of OS, we have demonstrated in this manuscript that hMSCs driven toward osteogenesis are not affected by chemotherapeutics (doxorubicin and cisplatin),
and hMSCs are still able to undergo differentiation into osteoblasts. Moreover, hMSCs seeded on MHA/Coll that undergo osteogenesis due to the unique properties of MHA/Coll demonstrate superior viability and superior osteogenic gene expression compared to hMSCs exposed to osteogenic media in the 2D condition. Therefore, we hypothesize that due to the highly osteogenic characteristic of MHA/Coll, endogenous hMSCs recruited to the scaffold can undergo osteogenesis even following surgical excision of OS in the presence of adjuvant chemotherapy.

There are some limitations of this study. We only evaluated the effect of doxorubicin and cisplatin on hMSCs and osteogenesis because we were unable to obtain methotrexate IC-50 value for either PDX-derived OS cells, which is supported by other groups.77

In addition, though our findings suggest that hMSCs seeded on MHA/Coll in the 2D environment undergo osteogenesis, making them less likely to migrate out of the 3D environment toward OS cells, we are unable to elucidate if hMSCs secrete cytokines that could potentially support tumor growth in a paracrine manner. However, our scaffold is unique compared to others utilized for bone regeneration; MHA/Coll is biomimetic that does not rely on the release of growth factors or stimulating bioactive molecules, therefore decreasing the chance of tumor recurrence and undesired side effects.

Also, we did not evaluate if OS cells migrate toward hMSC. Due to size limitations of MHA/Coll and the migration assay, it is not possible to determine if OS cells migrate toward hMSCs on MHA/Coll in the in vitro setting. A in vivo setting it will be ideal to determine if hMSCs secrete factors that would favor OS cell proliferation or migration.

Moreover, it has recently been reported that hMSCs effects are different based on the type of donor. Studies have suggested that hMSCs from healthy donors and hMSCs from patients who have OS could have a different endogenous response. Because our study utilized hMSCs from healthy patients, we cannot conclude that hMSCs from a donor with OS will demonstrate the same results. Additional experiments are warranted to determine the significance of the host hMSCs in this setting.

Another limitation of this study is related to the clinical complexity of OS.78,79 OS is most often located in the metaphysis of the long tubular bone with unclear edges and involves both new bone formation and bone destruction.80 X-rays often demonstrate trabecular bone destruction, and as the size of the tumor increases and expands through the cortex, the periosteum “flips” making the Codman triangle, a characteristic X-ray sign of OS. Though the Codman triangle is typical for OS but can also been seen in patients with osteomyelitis and Ewing’s sarcoma. Finally, in the late stage of OS growth, X-rays demonstrate a shadow of tumor infiltration into the soft tissue and as the size of OS can result in pathological fractures.

Though this is the first study to evaluate if osteogenesis is affected by chemotherapeutics in an 3D in vitro setting, this study did not include experiments that demonstrate the complex microenvironment of OS. However, due to the physical and chemical characteristics of MHA/Coll, the ability to determine the various cause and effects of OS cells, osteoblasts, macrophages, and other immune cells is difficult.81 MHA/Coll is florescent within the green florescent wavelength, causing a limitation of the ability to perform these complex experiments. Future experiments will need to be evaluated in vivo to determine how the interaction of MSCs, osteoblasts, and immune cells are affected by neoadiuvant and/or adjuvant chemotherapy. Our current results suggest that both neo-adjuvant and adjuvant chemotherapy should result in a modulation of the immune system that favors rapid bone formation and increased bone volume.

In conclusion, our study is the first one to demonstrate that a highly osteogenic scaffold could be oncologically safe since hMSCs recruited to MHA/Coll are able to undergo osteogenesis in vitro with decreased migration toward tumor cells without be affected by chemotherapy. We believe that in vivo, we will be able to further demonstrate that MHA/Coll shifts the balance of hMSCs between tumor recurrence and regeneration toward an environment that supports osteogenesis. The ability to repair bone voids following OS tumor resection utilizing a scaffold could greatly improve the clinical care, outcomes, and quality of life of patients with OS.

Materials and methods

All methods described were carried out in accordance with protocols approved by the Houston Methodist Institutional Review Board (IRB) to ensure the rights and welfare of human subjects’ protection during their participation compliance with the Code of Federal Regulations (45 CFR 46) established by Houston Methodist Research Institute with identification numbers CR00006624 and Pro00015718. Study participants provided written informed consent prior to the acquisition of samples.

Sample acquisition, cell isolation, and cell culture

Human bone marrow aspirate (h-BMA) was obtained from the orthopedic biorepository at Houston Methodist Hospital, which were previously obtained by the Department of Orthopedics and Sports Medicine Department (IRB CR00006624). The samples were individual and anonymously bio-banked. Samples were obtained from males and females, and the age ranged from 17 to 68 years old. Briefly, using sterile technique, 30 mL of bone marrow (BM) was aspirated from the proximal humerus, distal femur, or iliac crest. The aspirate was collected into a 30-mL syringe.
and Ham’s F-12 Medium (DMEM/F12 media, Gibco) grown in Dulbecco’s Modified Essential Medium (DMEM). Medium was changed every 72 h for 7, 14, and 21 days. Osteogenic differentiation was performed utilizing osteogenic differentiation media plus 10% osteogenesis supplement. Osteogenic differentiation was performed utilizing osteogenic differentiation media was made of αMEM media, (2) αMEM media plus one chemotherapy drug at IC-50 dose, (3) osteogenic differentiation media, or (4) osteogenic differentiation plus one chemotherapeutic at IC-50 dose. Osteogenic differentiation was performed utilizing osteogenic differentiation media was made of StemPro Osteocyte/Chondrocyte Differentiation Medium and 10% osteogenesis supplement. Media was changed every 72 h. At 7 and 21 days, media was aspirated and replaced with MTT resuspended in completed media at a concentration of 0.5 mg mL⁻¹. After 2 h, the MTT reagent was aspirated and replaced with an equal volume of DMSO. Following 30 min of gentle agitation at room temperature, absorbance was measured at 570 nm with reference wavelength of 630 nm using the Synergy H4 BioTek plate reader.

**IC50 of human TCCC-OS94 and TCCC-OS202 cell lines**

Human TCCC-OS94 or TCCC-OS202 cells were seeded on a 96-well plate. After 24 h, media was removed, and cells were treated for 72 h with increasing concentrations of methotrexate, doxorubicin, or cisplatin. The percentage of surviving cells relative to untreated controls was determined. The concentrations that inhibited cell growth by 50% (IC-50) were determined from each chemotherapeutic agent from logarithmic dose-response curves.

**Viability of hMSCs against human OS IC-50 chemotherapeutic doses**

Human MSCs (hMSCs) were seeded at 50,000 per well in a 24-well plate. At 75% confluence, cells were incubated with one of the four following conditions: (1) αMEM media, (2) αMEM media plus one chemotherapy drug at IC-50 dose, (3) osteogenic differentiation media, or (4) osteogenic differentiation plus one chemotherapeutic at IC-50 dose. Osteogenic differentiation was performed utilizing osteogenic differentiation media was made of StemPro Osteocyte/Chondrocyte Differentiation Medium and 10% osteogenesis supplement. Media was changed every 72 h. At 7 and 21 days, media was aspirated and replaced with MTT resuspended in completed media at a concentration of 0.5 mg mL⁻¹. After 2 h, the MTT reagent was aspirated and replaced with an equal volume of DMSO. Following 30 min of gentle agitation at room temperature, absorbance was measured at 570 nm with reference wavelength of 630 nm using the Synergy H4 BioTek plate reader.

**Porous Mg-doped type I collagen/hydroxyapatite (MHA/Coll) scaffold fabrication**

MHA/Coll functionalized scaffolds were fabricated from bovine tendon extracted type I Collagen using a freeze-drying method. About 200 g of type I collagen in acetic acid (5% w/v; Nitta Casings Inc., NJ, USA) were dissolved in 1 L deionized water at a final concentration of 10 mg mL⁻¹ in an aqueous acetic buffer solution at pH 3.5. Briefly, 40 mM aqueous solution of H₃PO₄ was added to 100 g of
the acetic collagen gel, and dropped in a solution of Ca(OH)\(_2\) (40 mM) and MgCl\(_2\)·6H\(_2\)O (2 mM) of deionized water. The material underwent a crosslinking in an aqueous solution of 1,4-butanediol diglycidyl ether (BDDGE) (2.5 mM), at 4°C for 24 h. After crosslinking, the slurry was washed once with distilled water.

**Casting of MHA/Coll scaffold**

After water rinsing, the final slurry was poured onto a 96-well culture plate and freeze-dried until the resulting porous scaffolds were formed. The scaffolds were sterilized by UV irradiation for 4 h under a laminar flow hood. The final porosity of the scaffold was generated by freeze drying. The material was briefly frozen from 20 to −20°C in 3 h and subsequently heated from −20 to 20°C in 3 h under vacuum conditions (80 mTorr).

**Casting of MHA/Coll membrane**

After water rinsing, the pH of the final slurry was adjusted by adding 50 mL of acetate buffer, washed, and then resuspended in 100 mL of H\(_2\)O. About 500 µL of glacial acetic acid was added to bring the pH to 4.5. Then 70 mL of slurry was cast in a lid and placed under the tissue culture hood and allowed to dry for 72 h by solvent casting.

**Size of MHA/Coll scaffold and membrane**

An 8 mm tissue biopsy punch was used to manufacture uniform MHA/Coll Membranes. MHA/Coll scaffolds were all cast in a 96 well plate and retained the shape of the well after freeze drying.

**Scanning electron microscopy (SEM)**

The morphology of the scaffold was characterized by scanning electron microscopy (SEM). Scaffolds were coated by 7 nm of Pt/Pl for scanning electron microscope (SEM; Nova NanoSEM 230, FEI, Hillsboro, OR, http://www.fei.com) and imaged using an accelerating voltage of 10 kV.

The volume of the pores was calculated by an ethanol infiltration method. (H. Tan, J. Wu, L. Lao, C. Gao, *Acta Biomater* 2009, 1, 328–337.) The volumes of MHA/Coll scaffold were measured by scaffold geometry (cylinders of 5 mm in diameter, 1 mm height).

The volume of the pores was defined by:

\[
V \rho = (W_e - W_0) / \rho_e
\]

Where \(W\) is scaffold’s weight before (W0) and after incubation in ethanol (We), and \(\rho_e\) (0.789 mg mL\(^{-1}\)) represents the ethanol density at room temperature.

The porosity of the scaffolds was calculated according to:

\[
P = \frac{V \rho}{V_s} \times 100
\]

**Rheology and compression testing**

Scaffolds of 0.5 cm thickness were soaked in PBS and loaded on UniVert Mechanical Test System. A Load Cell of 10N was calibrated and used to perform a compression test with maximum stretch magnitude of 35%, a stretch duration of 60 s and a recovery time of 60 s. A minimum of three replicates were performed and recorded for each condition. Each test stopped when compressive force limit was reached.

**Fourier transform infrared spectroscopy (FTIR)**

The samples were analyzed in transmission mode at resolution 4, 64 points, over the range of 2000–500 cm\(^{-1}\) using a Nicolet 6700 spectrometer (Thermo-Fisher Scientific, Waltham, MA, http://www.thermofisher.com). About 128 scans were performed with a resolution of four (H\(_2\)O and CO\(_2\) correction applied). Samples are normalized on AMIDE I peak.

**Thermal gravimetric analysis (TGA)**

The amount of mineral phase nucleated on the organic template (type I collagen) was quantified by thermal gravimetric analysis (TGA). The samples (\(n=3\)) were placed in alumina pans and subjected to a heating ramp from 25 to 800°C at 10°C min\(^{-1}\). A Q-600 TGA was used (TA Instruments).

**Viability of hMSCs on MHA/Coll membrane and MHA/Coll scaffold**

About 50,000 hMSCs in the 2D condition, 250,000 hMSCs on the MHA/Coll membrane, or 500,000 hMSCs on the MHA/Coll scaffold were seeded to evaluate viability of hMSCs that adhered to MHA/Coll. Prior to seeding hMSCs in the 3D condition, the MHA/Coll membrane or scaffold was placed in aMEM with FBS in the incubator for 45 min. After this time, excess aMEM media was removed and the appropriate number of cells were seeded on the membrane or scaffold in a volume of 20 µL. After 1 h, the MHA/Coll membrane or scaffold was transferred to a new well to ensure that hMSCs that had not adhered to MHA/Coll did not interfere with the experiment and fresh aMEM was added to the well. 48 h later, hMSCs from 2D cultures were recovered using trypsin whereas cells seeded into 3D were incubated with 500 µL of trypsin for 30 min at 37°C on a shaker and the supernatant was then removed and centrifuged for 10 min at 300G to collect hMSCs. The viability
of hMSCs was evaluated by LIVE/DEAD® (Life Technology) cell viability assay, which was performed according to manufacturer protocol by FACS Fortessa flow cytometer (BD Biosciences) and analyzed using FCS Express (Denovo Software).

Flow cytometry for hMSC cell surface markers

hMSC cells were analyzed with flow cytometry for MSC surface markers as defined by the International Society for Cellular Therapy. hMSCs were seeded at 50,000 per well in a 24-well plate in the 2D condition, 250,000 hMSCs on the MHA/Coll membrane, or 500,000 hMSCs on the MHA/Coll scaffold. About 2 days later, cells were incubated with either αMEM media, or osteogenic differentiation media. Media was changed every 72 h and cells were collected at 7 and 21 days. Briefly, cells from 2D cultures were recovered using trypsin whereas cells seeded into 3D were incubated with 500μL of trypsin for 30 min at 37°C on a shaker. The supernatant was then removed and centrifuged for 10 min at 300G.

Cells were washed with FACS buffer and stained for 30 min at 4°C with negative cocktail markers (human leukocyte antigen—DR isotype HLA-DR, CD45, CD11b, CD19, and CD34) and positive anti-human cocktail markers (cluster differentiation CD90, CD73, CD105). Conjugated primary monoclonal antibodies and isotype controls were used as recommended by the manufacturer (BD Biosciences). Cells were analyzed on a FACS Fortessa flow cytometer (BD Biosciences) and analyzed using FCS Express (Denovo Software).

The experiment was repeated in the presence of chemotherapeutics. Briefly, hMSCs were seeded in the 2D condition, on the MHA/Coll membrane and on the MHA/Coll scaffold were incubated with one of the following four conditions: (1) αMEM media, (2) αMEM media plus one of the chemotherapeutic drugs at IC50 value, (3) osteogenic differentiation media cells, (4) osteogenic differentiation media plus one of the chemotherapeutic drugs at IC-50 value. MSC surface markers were analyzed by flow cytometry as previously describe.

Osteogenic differentiation characterization

Osteogenic induction was confirmed by evaluating mineral deposition with von Kossa staining (Von Kossa Stain Kit American MasterTech). About 250,000 hMSCs on the MHA/Coll membrane, or 500,000 hMSCs on the MHA/Coll scaffold were seeded, controls included MHA/Coll membrane and scaffold with no hMSCs seeded. About 48 h later, calcium deposition was assessed.

Osteogenic gene expression analysis

Osteogenic differentiation was assessed in vitro at P4. hMSC were seeded at the density of 5000 cells cm⁻² in 12-well plates in the 2D condition and in the 3D condition on the MHA/Coll membrane. Confluence 2 days later, cells were incubated with αMEM media, or osteogenic differentiation media. For cell cultured in 2D, total RNA was isolated using 0.5 mL of Trizol reagent (Life Technologies, ThermoFisher Scientific) while for 3D cell cultured, 1 mL of trypsin was added to the scaffold/membrane and cells were detached after 30 min at 37°C on a shaker, then centrifuged for 10 min at 300G. Following hMSC isolation, 1 mL of Trizol was added. Samples were mixed with 100 mL chloroform (Sigma-Aldrich, MI, USA) and incubated at RT for 2 min. A centrifugation cycle for 15 min at 12,000 g and 4°C was performed to separate the RNA aqueous phase. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. Samples were incubated at room temperature for 10 min and then centrifuged for 10 min at 4°C. The supernatant was removed, and the RNA pellet was washed with 75% ethanol. As a final step, the RNA was eluted in 20 μL of RNase-free water and quantified using a ND1000 spectrophotometer (ND1000, NanoDrop®, ThermoFisher Scientific, MA, USA). The cDNA was synthesized from 1 μg of total RNA using iScript™ cDNA synthesis kit (Bio-Rad, CA, USA). Amplifications were set on plates in a final volume of 10 μL and carried out using TaqMan Fast Advanced MasterMix (Applied Biosystems, ThermoFisher Scientific) using StepOneTM Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific, MA, USA). The housekeeping marker included in the study was eukaryotic 18S rRNA (18S; Hs03003631_g1). The specific osteogenesis lineage associated markers used were: osteocalcin (BGLAP; Hs01587814_g1), alkaline phosphatase (ALP; Hs01029144_m1), osteopontin (SPP1; Hs03003631_g1). Relative gene expression was determined using data from the real-time cycler and the ∆∆CT method.

The experiment was repeated in presence of chemotherapeutics. Briefly, hMSCs were seeded in the 2D condition, on the MHA/Coll membrane and on the MHA/Coll scaffold were incubated with one of the following four conditions: (1) αMEM media, (2) αMEM media plus one of the osteogenic differentiation media cells, (3) osteogenic differentiation media plus one of the chemotherapeutic drugs at IC-50 value, (4) osteogenic differentiation plus one osteotherapeutic at the IC-50 dose. At different time points (7 and 21 days) differentiation was evaluated by the expression of osteogenic genes as previously describe.

Migration assay

Prior to seeding hMSCs, the MHA/Coll membrane or scaffold was placed in αMEM in the incubator for 45 min. After this time, excess αMEM media was removed and the appropriate number of cells were seeded on the membrane or scaffold in a volume of 20 μL 250,000 hMSCs on the MHA/Coll membrane and 500,000 hMSCs on the MHA/Coll scaffold were used. After 1 h, the MHA/Coll membrane or scaffold in αMEM media was transferred to a new well to ensure that hMSCs that had not adhered to
MHA/Coll did not interfere with the experiment and fresh aMEM was added to the well. For 2D condition 50,000 hMSCs were used. After 48 h, hMSCs from 2D cultures were recovered using trypsin whereas cells seeded into 3D were incubated with 500 μL of trypsin for 30 min at 37°C on a shaker and the supernatant was then removed and centrifuged for 10 min at 300G to collect hMSCs.

For the migration potential evaluation, isolated hMSCs from each condition (2D, MHA/Coll membrane, MHA/Coll scaffold) were then seeded on a transwell insert (8 μM pore size). At the bottom of the 12 well plate, either PDX-derived OS cells (TCCC-OS94 or TCCC-OS202) or the conditional media from each cells line was placed. As control we used 2D condition either aMEM or osteogenic media in the bottom well. After 12 h, the transwell inserts were washed with PBS, and cells that had not migrated from the top to the bottom of the well were removed. Cells that had migrated through the transwell were fixed in 70% ethanol. After drying, cells were stained with crystal violet for 5 min, washed in PBS, and then imaged using the Keyence microscope in three different views at 4× and 10×. The number of hMSCs that migrated through the transwell was counted and averaged for each condition.

The experiment was repeated in presence of chemotherapeutics. hMSCs in the 2D or 3D condition were incubated with one of the four following conditions: (1) αMEM media, (2) αMEM media plus one chemotherapy drug at the IC-50 dose, (3) osteogenic differentiation media, or (4) osteogenic differentiation plus one chemotherapeutic at the IC-50 dose. At the bottom of the 12 well plate, either PDX-derived OS cells (TCCC-OS94 or TCCC-OS202) or the conditional media aMEME or osteogenic media was placed. After 12 h, as previously described, cells were fixed, stained, imaged, and counted.

**Statistical analysis**

All experimental data distributions were assessed for normality using the Kolmogorov Smirnov test. Data with a normal distribution, t-tests, one-way and two-way Analysis of Variance (ANOVA) tests (GraphPad Prism 9, CA, USA) were used to determine significant differences between groups. The test statistic and corresponding p value were reported, and statistical significance defined as p < 0.05. All statistical analysis for each data set described is listed in the figure legends.

**Availability of data and materials**

All data are available in the main text or supplementary material.

**Author contributions**

AB: Conception and design, analysis and interpretation of data, drafting of article, collection and assembly of data, SL: Conception and design, critical revision of article for important intellectual content, collection and assembly of data, FP, SS: collection and assembly of data, PM: Conception and design, provision of study materials or patients, JY, BW: Conception and design, critical revision of article for important intellectual content, FT: Conception and design, analysis and interpretation of data, critical revision of article for important intellectual content.

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**Informed consent, ethical approval, human rights**

Research was conducted in accordance with the World Medical Association Declaration of Helsinki. Protocols were approved by the Houston Methodist Institutional Review Board (IRB) to ensure the rights and welfare of human subjects’ protection during their participation compliance with the Code of Federal Regulations (45 CFR 46) established by Houston Methodist Research Institute with identification numbers CR00006624 and Pro00015718. Study participants provided written informed consent prior to the acquisition of samples.

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**Supplemental material**

Supplemental material for this article is available online.

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