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Anatomical study of an unusual variant of the great saphenous vein in cadaver: case report and nomenclature review

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Abstract. During routine cadaveric dissection for undergraduate medical students, a variant of the great saphenous vein (GSV) was revealed. Its peculiarity resides in the relationship with the deep aponeurotic fascia in the thigh (i.e. fascia lata). Particularly, the GSV duplicated in two vessels at knee level: a "superficial" vein ran in the saphenous compartment (between superficial and deep aponeurotic fasciae), while the other deepened below the fascia lata. Given the clinical importance of the GSV and its frequent anatomical variations, this case report gave us the cue for reviewing the normal anatomy of GSV and for a critical revision of the nomenclature of its anatomical variations. GSV variants can be divided into two main patterns: i) the presence of a parallel accessory vein, and ii) vein duplication. The main features to distinguish these two variations reside in the caliber of the vein and its position with respect to the saphenous compartment. When compared to variants reported in scientific literature, our case seemed not to completely fit neither with the definition of a duplicated vessel or of an accessory vein. Remarkably, the presence of GSV variant may have key clinical implications. In particular, the relationship with fascial layers could influence the development of vein dilation and varicosity. Therefore, the understanding of GSV anatomy and its accurate study by ultrasound seem to be essential to support clinical and surgical practices relating to the superficial venous drainage of the lower limb.

Keywords: great saphenous vein, anatomical variant, fascia lata, duplication, accessory veins.

CASE REPORT

An anatomical variation of the great saphenous vein (GSV) was found in the left lower limb of a 108-year-old male cadaver during routine cadaveric dissection for undergraduate medical students. The left GSV had a normal origin from the dorsal venous arch of the foot, anterior to the medial malleolus and ascending the leg along its anteromedial surface, superficially to the crural fascia (Figure 1).

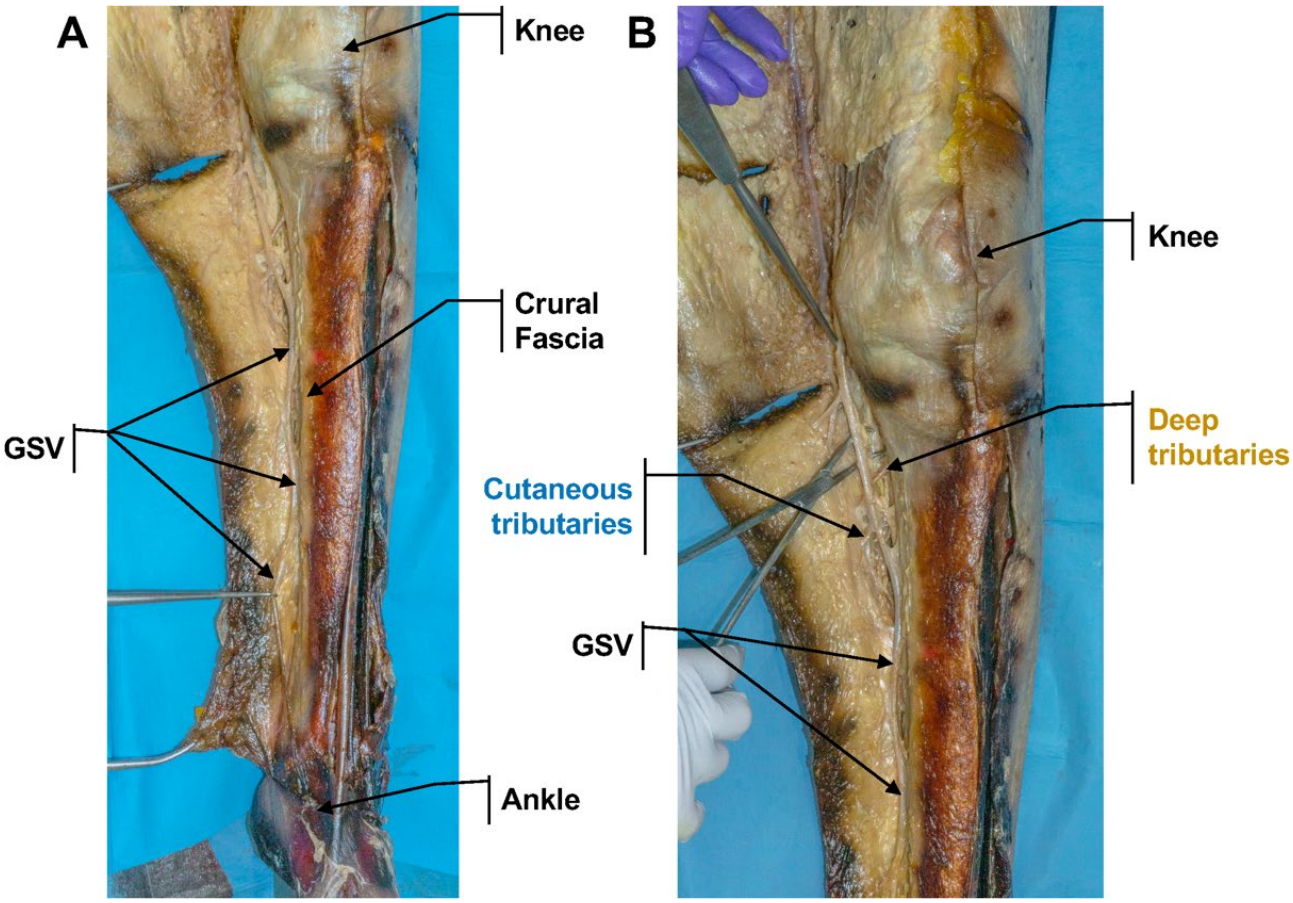

Figure 1. Course of great saphenous vein (GSV) in the leg. A. The course of the left GSV in the leg is shown. The GSV is hooked to expose its position with respect to the crural fascia. B. The GSV is hooked to expose cutaneous and deep tributaries.

At the medial condyle of the tibia, a venous vessel was individuated posteromedially to the GSV and originating from the subcutaneous layer (Figure 2).

This vessel had the same diameter of the GSV, and an anastomosing branch connected the two vessels. After the anastomosis, the two veins ascended individually and parallelly to each other. The one initially individuated in the leg deepened below the fascia lata; the other ran superficially into the saphenous compartment (Figure 3). The two vessels kept the same diameter as they ascended in the thigh region. The right lower limb did not present the same variation.

Given the complexity of the examined variation, we introduced the following nomenclature for the two veins individuated in the thigh:

– Superficial GSV (sGSV): the superficial venous vessel which lies in the saphenous compartment of the thigh and originates at the medial condyle of the tibia;
– Deep GSV (dGSV): the continuation of the GSV in the thigh, running underneath the fascia lata.

To expose the dGSV, the fascia lata was opened at the knee and partially removed during the dissection. In the thigh region, the fascia lata was cut on the midline and medially lifted in order to appreciate its relationship with the GSVs. At the upper one-third of the surface of the thigh, the dGSV pierced the fascia lata and joined the superficial vessel to form a single vein (Figure 4) which entered the saphenous hiatum, hence draining into the femoral vein. Notably, the dGSV received perforating veins from the deep veins of the limb, while the sGSV presented tributary branches from the cutaneous and subcutaneous layers (Figure 3).

Given the clinical importance of this vessel and its frequent anatomical variations, this case report gave us the cue for reviewing the normal anatomy of GSV and for a critical revision of the nomenclature of its anatomical variations.

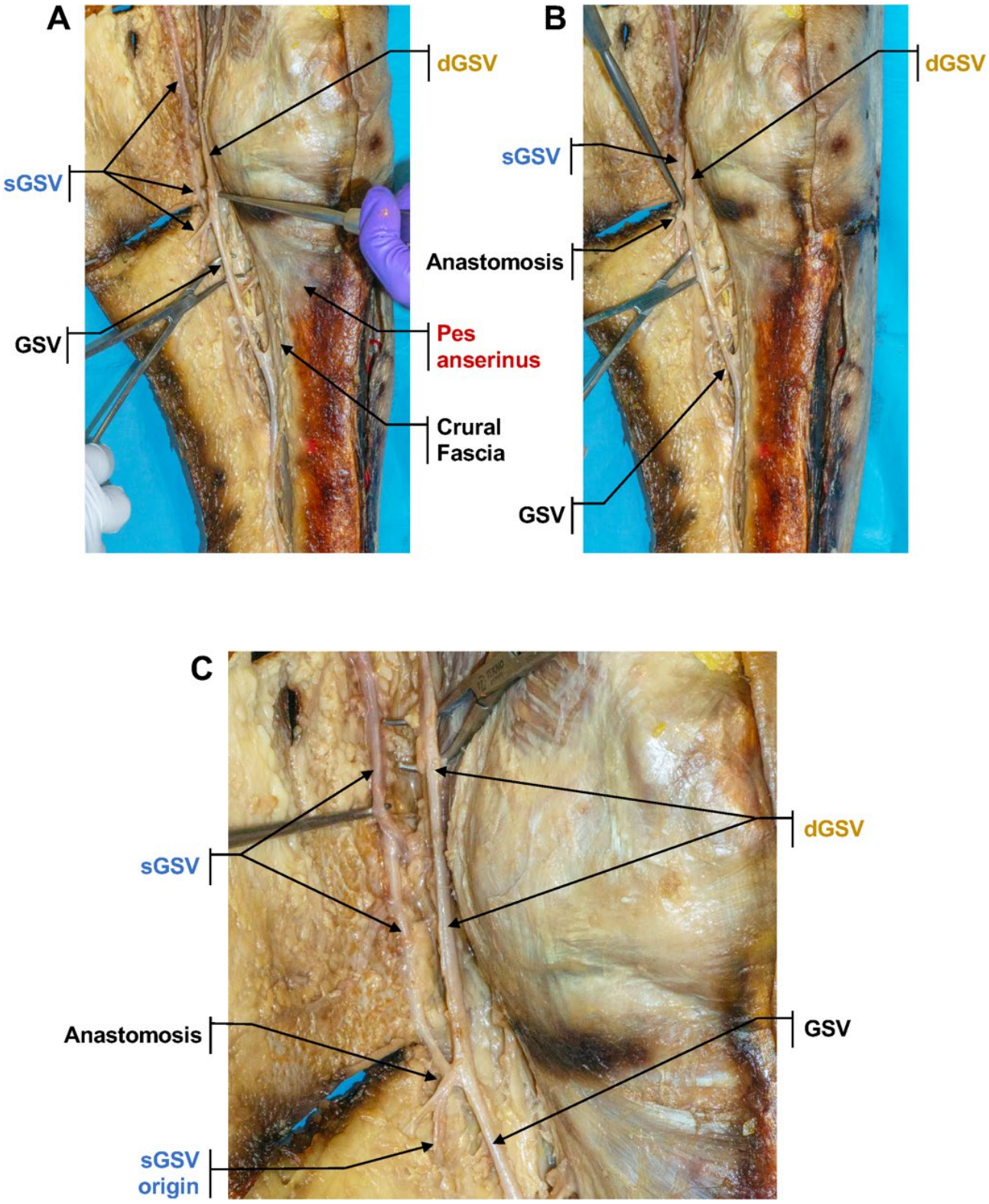

Figure 2. Origin of the "superficial" great saphenous vein (GSV) and anastomosis with the "deep" GSV. A. The origin of the superficial GSV (sGSV) at the level of the medial condyle of the tibia and posteromedially to the deep GSV (dGSV) is shown. B. An anastomosing branch connecting the two vessels is exposed. C. Magnification of the sGSV origin and its anastomosis with the dGSV.

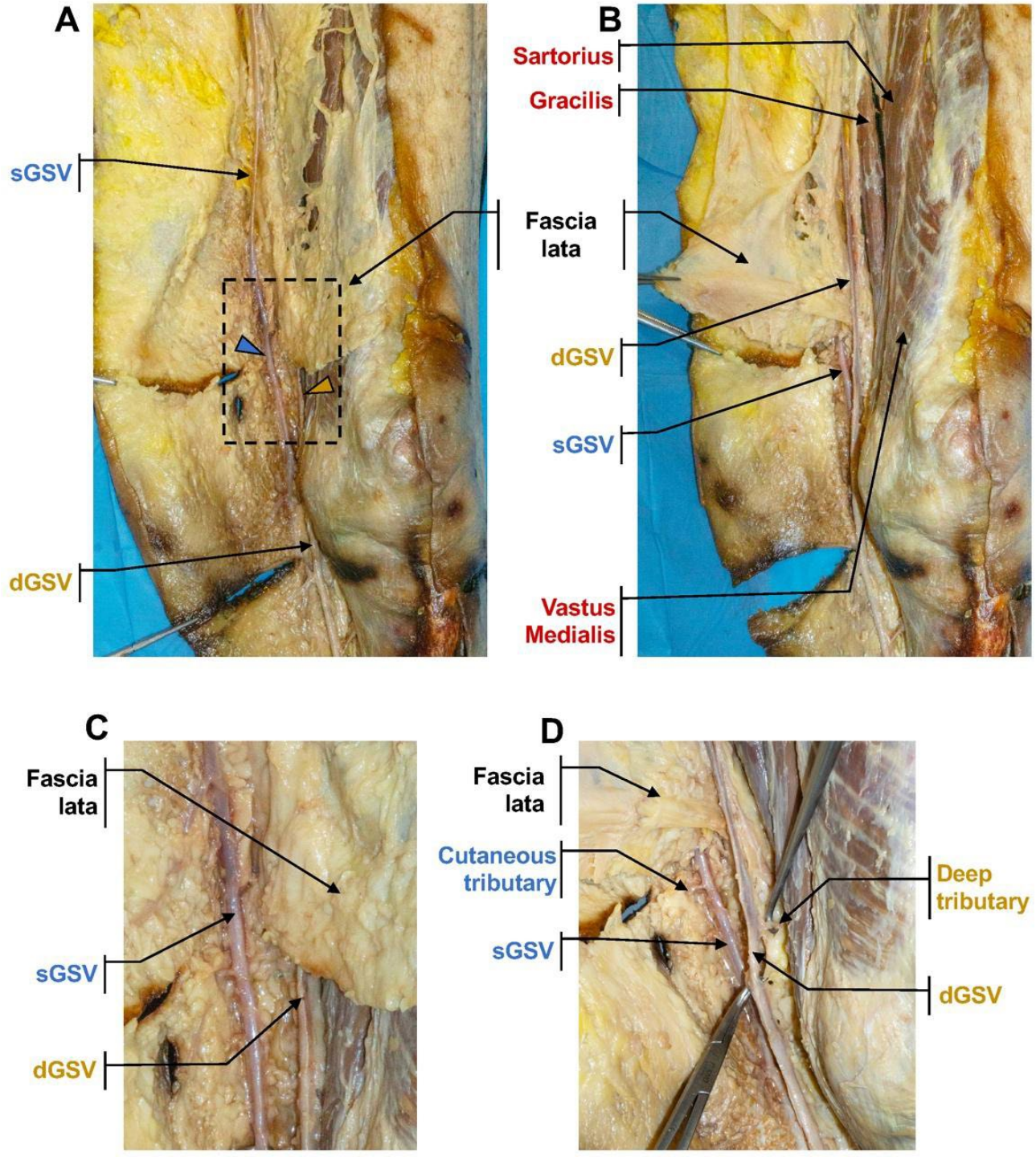

Figure 3. Relationship between the great saphenous veins (GSV) and the fascia lata. A. The GSV initially individuated in the leg deepened below the fascia lata (deep GSV – dGSV, yellow arrowhead); the other ran superficially within the saphenous compartment (superficial GSV – sGSV, blue arrowhead). B. The fascia lata was cut on the midline and medially lifted in order to expose the dGSV course. C. Magnification of the box in panel A. D. The GSV is hooked to expose deep tributaries joining the dGSV and cutaneous tributaries joining the sGSV.

Figure 4. Terminal course of the superficial and deep great saphenous veins (GSV) in the thigh. A. The superficial GSV (sGSV) is hooked to demonstrate the point where the deep GSV (dGSV, red arrowhead) pierces the fascia lata and joins the superficial GSV (sGSV) to form a single vein. B. The fascia lata was cut on the midline and medially lifted in order to expose the dGSV course. C. Magnification of the box in panel A.

NOMENCLATURE REVIEW

Normal anatomy of the great saphenous vein

The great saphenous vein, also known as long saphenous vein, is a large blood vessel responsible for most of the superficial venous drainage of the lower limb(Caggiati et al. 2002). Its origin can be found superficially at the medial portion of the dorsal region of the foot about 2-3 cm to the front of the medial malleolus; there, the GSV arises as the continuation of the medial marginal vein of the foot and runs within the subcutaneous layer above the dorsal fascia of the foot(Becciolini et al. 2021). Once it passes the medial malleolus, the great saphenous vein proceeds (still subcutaneously) along the medial surface of the leg, accompanied by the tibial branch of the saphenous nerve(Meissner 2005). Then, the GSV continues its course running posteriorly to the medial condyle of the femur; here, it receives anastomotic branches from the small saphenous vein before the latter drains into the popliteal vein(Tuveri et al. 2009).

In the thigh, the GSV proceeds above the fascia lata, following the course of the sartorius muscle and collecting vessels of the superficial venous network. The GSV runs in the subcutaneous layer throughout the thigh. At the femoral triangle (3-4 cm below the inguinal ligament), it perforates the cribriform fascia to join the femoral vein(Souroullas et al. 2017).

Along its course, GSV is typically located within the so-called saphenous compartment, which is demarked by the superficial and aponeurotic deep fasciae(Chen and Prasad 2009). Moreover, it receives multiple tributaries from the deep venous system (i.e. the perforating veins), which are crucial in balancing blood flow during muscle contraction. Four crucial perforator groups have been identified: upper thigh (Hunterian), lower thigh (Dodd's), at knee level (Boyd's) and in the calf region (Cockett's)(Cina et al. 2005).

Definition of GSV anatomical variations

The GSV may present variations in terms of course, relations to the fascia, and possible duplications of the vessel(Black 2014; Chen and Prasad 2009; Ricci and Cavezzi 2002). Previous literature developed different classifications concerning the GSV's variations(Chen and Prasad 2009). In summary, GSV variants could be divided into two main patterns: i) vein duplication and ii) the presence of a parallel accessory vein.

GSV duplication. Specific criteria must be met to classify a variation as a duplication. A duplication should present as two vessels that lie in the same plane, parallel to the skin and within the saphenous compartment, between the superficial fascia and the aponeurotic deep fasciae (i.e. crural fascia and the fascia lata, respectively in the leg and in the thigh)(Ricci and Caggiati 1999; Kumar et al. 2017). Moreover, the two GSVs should have similar caliber and should drain a common cutaneous territory(Kockaert et al. 2012). The two vessels can frequently present communicating branches and can have a complete course along the lower limb. Duplicated vessels can join together before draining into the femoral vein or, alternatively, can open separately into the femoral vein(Chen and Prasad 2009).

According to Chen and Prasad, duplication incidence is reported in 1% of cases(Chen and Prasad 2009). However, literature reports a range from 1% to 20% of duplication of the GSV. Kockaert M et al. indicated that authentic duplications of the GSV are less frequent than reported in literature, specifically 1.6% to 2%(Kockaert et al. 2012). Combining the retrospective duplex ultrasound and the prospective duplex ultrasound studies, Kockaert et al. indicated that true duplication of the GSV occurs in 1.8% of the duplex examinations of the GSV. GSV's duplication is more frequent in male than female individuals, is more common unilaterally than bilaterally and in the thigh compared to the leg(Corrales et al. 2002).

Kumar et al. reported a unique variation of the GSV. In the cadaver they studied, the GSV followed its regular path in the lower two-thirds of the leg(Kumar et al. 2017); upon reaching the upper one-third of the medial aspect of the leg, it bifurcated into a thick anterior and a thinner posterior trunk that ascended parallel to each other. The two vessels merged together at the upper third of the thigh. Given the difference in size and caliber, the authors preferred to use the term "bifurcation" rather than "duplication".

Parallel accessory vein. An accessory vein running in parallel with the GSV is usually smaller in size and does not drain the same cutaneous territory as the GSV(Cotton 1961; Caggiati 2000; Padavinangadi et al. 2015). Generally, the accessory vein runs superficially to the saphenous compartment. In some cases, the accessory vein can appear large and thick and may enter the saphenous compartment by piercing the superficial fascia(Chen and Prasad 2009). Schematically, an accessory vein could present with three most frequent patterns in the thigh(Chen and Prasad 2009; Ekin and Kurtul Yildiz 2017; Oguzkurt 2012):

i) A large subcutaneous tributary runs superficially to the saphenous compartment and pierces the superficial fascia to join the GSV at a variable level in the thigh.

ii) Proximally, the GSV lies within saphenous compartment and, distally, a large accessory vein is present in the subcutaneous layer with no other substantial vein visible in the saphenous compartment. The accessory vein pierces the saphenous fascia at a variable level in the thigh to join the GSV.

iii) An accessory saphenous vein runs together with the GSV below the superficial fascia into separate saphenous compartments; the two veins then join and lie within a single saphenous compartment before entering the saphenofemoral junction. In the latter situation, the accessory saphenous vein could be mistaken as a duplication of the GSV, especially at ultrasound examination.

In summary, in the presence of GSV variations and for an accurate classification, attention should be pointed at the caliber of the vein and its position with respect to superficial (saphenous) fascia(Caggiati 1999; Caggiati and Ricci 1997). The GSV runs in a deep position (so-called "saphenous compartment") while accessory veins are in a superficial plane. Evidenced of this can be obtained by ultrasonography or computed tomography, and it can be considered a useful interventional and diagnostic indicator that can lead to the individuation of the saphenous vein from a large parallel accessory vein.

Echo-anatomy of the GSV

Duplex ultrasonography is a widely used tool to evaluate the venous system anatomy and determine possible variations especially in the clinical practice.

In the proximal thigh, the GSV can be individuated thanks to its typical appearance known as the "saphenous eye" or "Egyptian eye" in a transverse scan(Ricci and Caggiati 1999). This peculiar depict is due to its position within the saphenous compartment, demarked by the superficial and the aponeurotic deep fasciae.

Due to the presence of numerous tributaries, it may be difficult to identify the "saphenous eye" at the knee level. As a matter of fact, the "tibio-gastrocnemius angle" sign has been demonstrated to be a useful tool in the detection of the GSV in the knee area within the saphenous compartment(Oguzkurt 2012). The tibio-gastrocnemius angle is delimited by the tibia, the medial head of

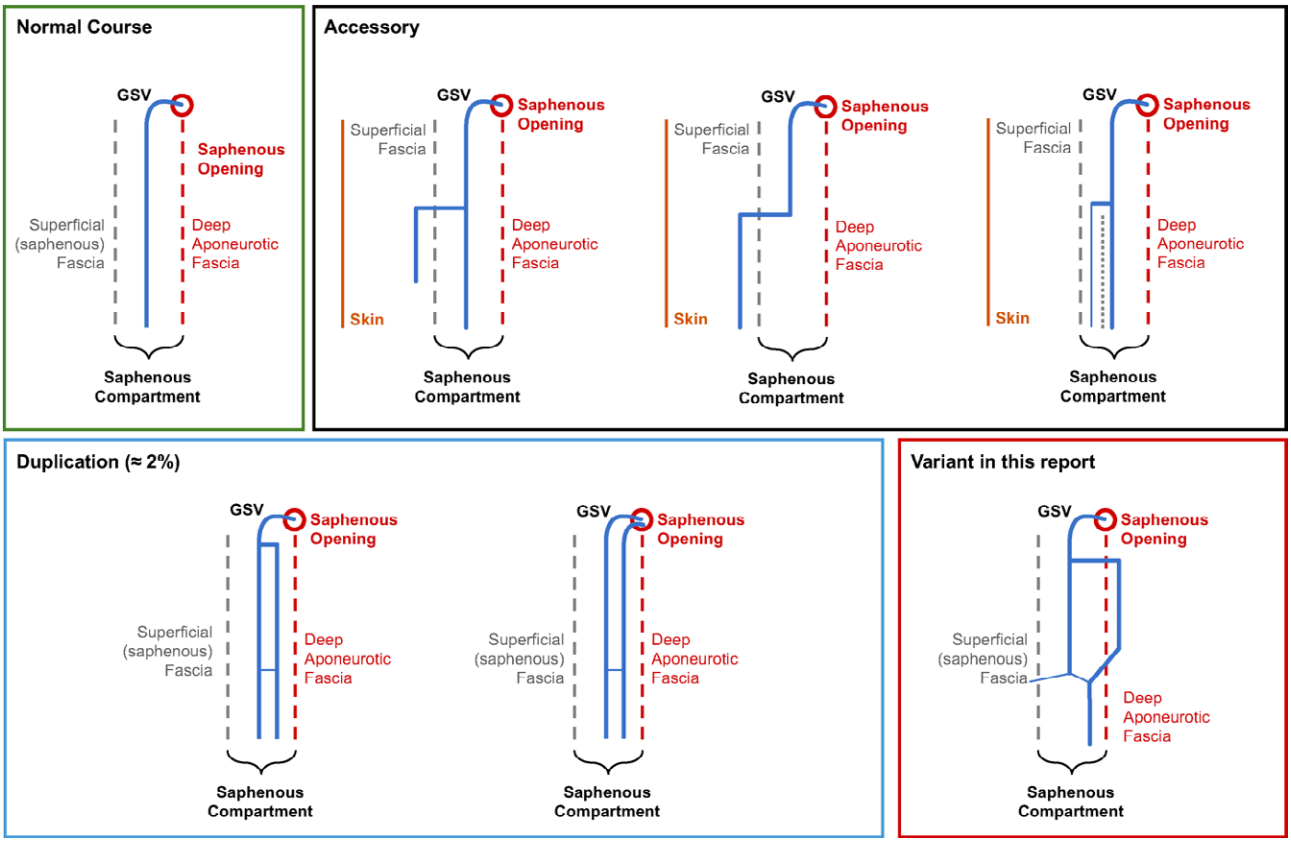

Figure 5. Schematic representation of great saphenous vein (GSV) anatomy and its variants. Normal course, accessory vein patterns, and duplications are represented and compared to the variant reported in this paper.

gastrocnemius muscle and the saphenous fascia. Of note, the GSV may be absent in this triangle in case of segmental hypoplasia of the GSV or if a segment of the GSV is located outside the saphenous compartment(Chen and Prasad 2009).

Moreover, another ultrasound marker for the GSV at the thigh level was described as the "E-point"(Ricci et al. 2017). This marker represents a very superficial position of the GSV which can be tracked in cross-section starting from the sapheno-femoral junction and optimally visualized where it crosses the adductor longus muscle, 3–5 cm below the junction.

The GSV variants and clinical implications

Varicose veins and valvular reflux. Varicose veins are twisted, dilated veins mostly located in the lower extremities(Jones and Carek 2008). Nearly 33% of the general population aged between 18 and 64 is affected by varicose veins(Aslam et al. 2022).

In the lower limb, the venous returns is sustained by three main factors: the muscle pump(Recek 2013; Uhl and Gillot 2015), venous valves(Goldman and Fronek 1989; Moore, Gohel, and Davies 2011), and perforating veins(Baliyan et al. 2016). Alterations in vein valves is pivotal in determining venous reflux and varicosity. The valvular dysfunction is presumed to be caused by a loss of elasticity in the vein wall, with incomplete valve closure and consequent valvular reflux(Clarke et al. 1992; Raetz, Wilson, and Collins 2019).

Venous reflux may occur along the course of the great saphenous vein. In this condition, the GSV often expands and enlarges its diameter: venous dilation may be variable based on the reflux entity and location along the GSV course(Kim et al. 2020; Thomson 1979). However, venous reflux and varices in the lower limb are not always associated to an incompetent or dilated GSV. It has been suggested that the presence of a large accessory vein or vein duplication could predispose to varicosity and reflux. Remarkably, the pathogenetic mechanisms leading to vein incompetence could be enhanced in accessory veins or duplications because these anatomical variants could have peculiar histological features(Caggiati 2000).

Hence, subjects with anatomical variants of the GSV may have a congenital predisposition to varicose vein disease based on relationships with fascial components. Accordingly, superficial and aponeurotic deep fasciae protect GSV within the saphenous compartment; differently, a parallel accessory vein lacks this fascial sheath, thus predisposing to vein incompetence(Ricci and Cavezzi 2002).

GSV's clinical relevance in bypass surgery. The GSV represents main venous conduit used for patients undergoing coronary artery bypass grafting(Akca et al. 2020). Furthermore, the GSV is used in infrainguinal bypass surgery(Belvedere et al. 2020). In this context, in case of bypass for critical limb ischemia, the use of the GSV is reported to be superior compared to alternative autologous vein bypass(Arvela et al. 2012). It is interesting to note that the GSV can be also effectively and safely used as a temporary bypass in long and invasive surgical procedures.

In this light, surgeons must be aware of anatomical variants of the GSV, especially when considering duplications. The removal of GSVs may result in iatrogenic varicosity of the lower limb, particularly when accessory veins are present. As mentioned before, the accessory vessels often have a structural and histological predisposition to varicosity; also, the surgical removal of a duplicated GSV may increase the risk of venous reflux and the development of varices.

CONCLUSIONS

The reported anatomical variation of GSV is peculiar and seems to not completely fit with any conventional variations described above.

Similarly to GSV duplications, the two vessels (sGSV and dGSV, Figure 5 and 6) have the same caliber and ascend parallel to each another; however, they do not lie on the same plane and do not drain a common territory.

Uniquely, the superficial vessel (sGSV) resides in the saphenous compartment, while the deep one (dGSV) lies underneath the fascia lata. Moreover, they seem to have different drainage territories: sGSV receives tributaries from the subcutaneous layers of the thigh, whereas dGSV is reached by perforating (deep) veins. Finally, the two veins do not have the same origin. Based on these features, the observed variant does not fulfill the criteria for considering it as a duplication(Chen and Prasad 2009; Padavinangadi et al. 2015). Moreover, the sGSV cannot be defined as an accessory vein either. Generally, accessory veins have a smaller caliber compared to the GSV and do not run into the saphenous compartment(Ricci and Cavezzi 2002; Caggiati 2000).

Remarkably, the GSV has a conventional configuration in the leg, lying within the saphenous compartment and draining the superficial layers of this region. Just above the tibial tuberosity, the vessel pierced the aponeurotic deep fascia and continued as the dGSV. Parallel, at this level, the sGSV emerged from the subcutaneous layer and ascended within the saphenous compartment in the thigh. In the proximal thigh, the dGSV pierces the

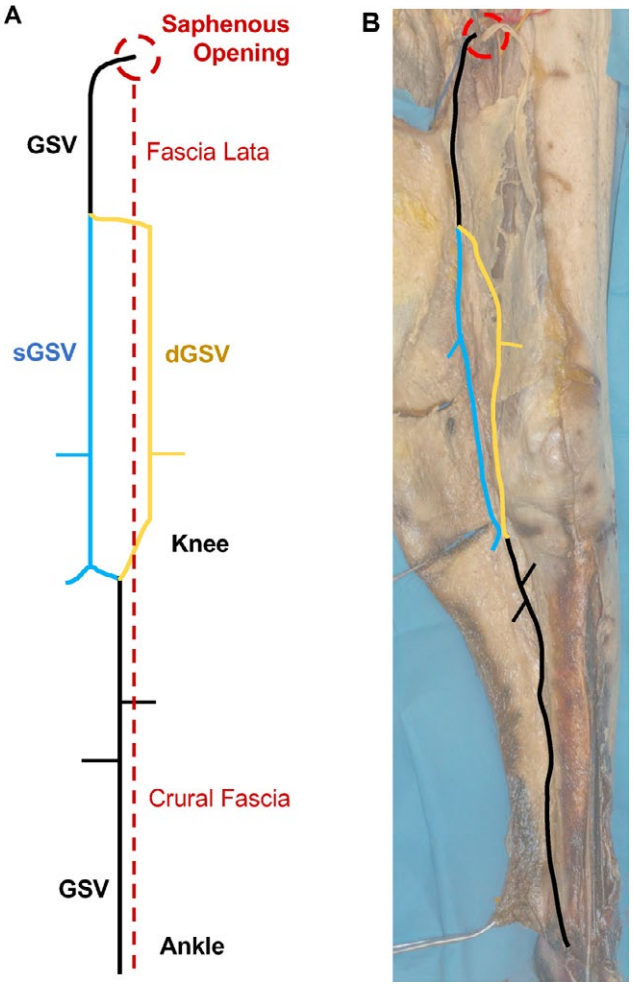

Figure 6. Summary of the described variant of the great saphenous vein (GSV) and its course along the lower limb. A. Schematic representation of the reported GSV variant and its relationship with the crural fascia in the leg and fascia lata in the thigh. sGSV: superficial GSV; dGSV: deep GSV. B. The schematic representation of the reported GSV variant is superimposed onto the cadaver image.

fascia lata and merged with the sGSV before draining into the femoral vein. Therefore, the peculiarity of this variation could affect its recognition at ultrasound examination. This could have an impact on the evaluation of eventual clinical implications; moreover, the actual incidence of the observed variation might be higher.

Although anatomical variants could predispose to venous reflux due to their relationship with fasciae(Ricci and Caggiati 1999; Thomson 1979), the subject did not show any varicosity; this is in agreement with the fact that both vessels are anatomically associated to an anatomical fascia, thus preventing excessive dilation.

In conclusion, we described an unusual anatomical variation of the GSV characterized by a peculiar relationship with the fasciae of the lower limb. Given the frequency and clinical implications of GSV variations, this case report furnished the opportunity to review the conventional anatomy of GSV and to summarize the most common variations in its course. An accurate ultrasound study of GSV is essential to support clinical and surgical practices relating to the superficial venous drainage of the lower limb.

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Unusual pulmonary vein variations: A new case report and its clinical significance

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Abstract. Anatomical variations in pulmonary circulation, especially in the number and drainage patterns of pulmonary veins, have significant clinical implications. The typical anatomy includes four pulmonary veins, which transport oxygenated blood to the left atrium through distinct ostia. However, variations have been reported in up to 36% of cases, a discovery facilitated by advances in imaging and cadaveric dissection. This study presents the anatomical findings of supernumerary pulmonary veins observed during routine thoracic dissection of a 75-year-old female cadaver. The implications of such variations are critical in cardiothoracic and pulmonary surgery, where unrecognized anatomical differences may lead to complications, including intraoperative bleeding and incomplete resections. Knowledge of these variations is also pivotal to radiological interpretation, as misinterpretation can lead to diagnostic errors. This study emphasizes the need for clinicians to be aware of pulmonary venous variability to enhance surgical planning, reduce procedural risks, and improve patient outcomes.

Keywords: pulmonary veins, anatomical variation, left atrium, supernumerary pulmonary vein, cadaveric dissection, clinical anatomy.

INTRODUCTION

It is widely known that the human vasculature commonly exhibits variable patterns in many organs, such as the kidneys [1], aortic arch [2], and thyroid gland [3]; these are therefore regarded as "normal" variations [4].

Regarding anatomical variations in the pulmonary circulation, variability in pulmonary venous ostial anatomy was previously considered rare and documented only in a few case reports [5].

The "normal" anatomy comprises four pulmonary veins, two from each lung, which return oxygenated blood to the left atrium, with each vein opening through a separate ostium (Figure 1). Since the second half of the 20th

century, different patterns in the number of pulmonary veins and left atrial ostia have been reported, and these numbers do not always correspond [5]. Accordingly, contrary to earlier assumptions, recent studies have reported variations in pulmonary venous anatomy in approximately 36% of cases. This increased recognition has been facilitated by advances in imaging, non-invasive diagnostic techniques, conventional angiography, and cadaveric dissection [6]. Variations in number and drainage patterns have recently gained increasing attention not only in cardiothoracic surgery and pulmonary lobectomy, because pulmonary veins play an important role in determining segmental lung anatomy [7], but also in interventional procedures such as radiofrequency ablation for atrial fibrillation. Indeed, pulmonary venous variability has been associated with ectopic cardiac activity [8]. The present study aims to highlight the importance of knowledge of pulmonary venous anatomy, focusing on the presence of supernumerary pulmonary veins and their drainage into the left atrial ostia.

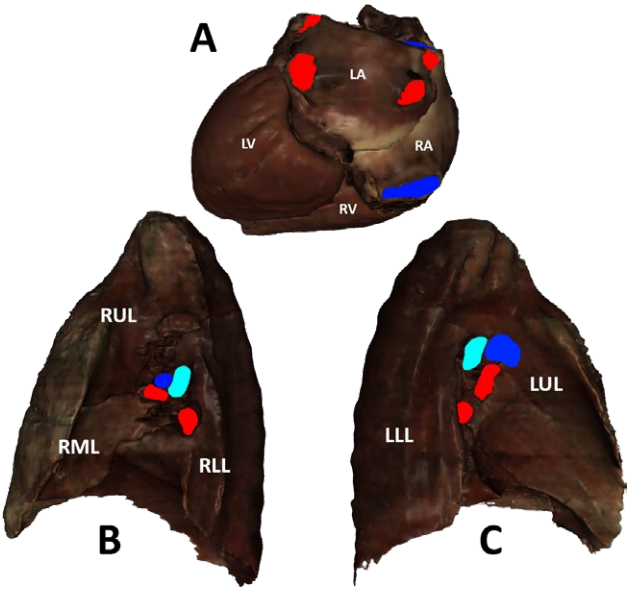

Figure 1. *Heart and lungs showing the "normal" anatomy of the arteries, veins, and bronchi.* (A) The dorsal surface of the heart *in situ*, with four pulmonary vein ostia highlighted in red, and the superior and inferior venae cavae highlighted in blue. (B) The mediastinal surface of the right lung shows, at the hilum, the right bronchi (cyan), right pulmonary artery (blue), and two right pulmonary veins (red). (C) The mediastinal surface of the left lung shows, at the hilum, the left bronchi (cyan), left pulmonary artery (blue), and two left pulmonary veins (red). LA=left atrium; RA=right atrium; LV=left ventricle; RV=right ventricle; RUL=right upper lobe; RML=right middle lobe; RLL=right lower lobe; LUL=left upper lobe; LLL=left lower lobe. The image was adapted from "SECTRA Education Portal, Dissector Atlas v. 6.3.5, epsectra.com. Dissector Atlas is powered by Virtual Human Dissector and provided by Touch of Life Technologies".

MATERIALS AND METHODS

The anatomical variability of the pulmonary veins was observed in a 75-year-old female cadaver. The specimen was obtained during routine undergraduate thoracic dissection classes held at the ICLO Teaching and Research Center (Verona, Italy), under the supervision of dissection technicians, clinicians, and professors.

The unfixed lung was resected at the hilum, close to the vascular and bronchial pedicles. Any adhesions between the parietal and visceral pleura were removed by hand.

Consent for the use of the self-donated cadaver in this study was obtained from the donor before death.

The study protocol conformed to the guidelines set out by the Declaration of Helsinki.

DISCUSSION

Four pulmonary veins, two superior and two inferior, typically drain oxygenated blood from the lungs into the left atrium through distinct ostia. However, anatomical variations in the number of pulmonary veins can occur. While these variations are often clinically silent, an in-depth understanding of pulmonary venous anatomy and its relationship to the left atrium is essential. This knowledge is critical not only for pre-procedural planning in thoracic and cardiac surgery [10], but also for understanding the association between pulmonary vein anomalies and atrial fibrillation [9].

In addition to radiofrequency ablation for atrial fibrillation, other clinical scenarios demonstrate the significance of pulmonary vein variations. For instance, during pulmonary lobectomies, where the aim is to remove lung segments affected by cancer, knowledge of supernumerary pulmonary veins can help avoid intraoperative complications, such as inadvertent venous injury or excessive bleeding. Misidentification of these vessels may lead to incomplete resections or compromised postoperative lung function. Similarly, in lung transplantation procedures, surgeons must be aware of variations in venous anatomy to ensure successful anastomosis and proper venous drainage, minimizing the risk of graft failure or postoperative complications such as pulmonary vein thrombosis.

Furthermore, variations in pulmonary venous anatomy can affect the interpretation of diagnostic imaging,

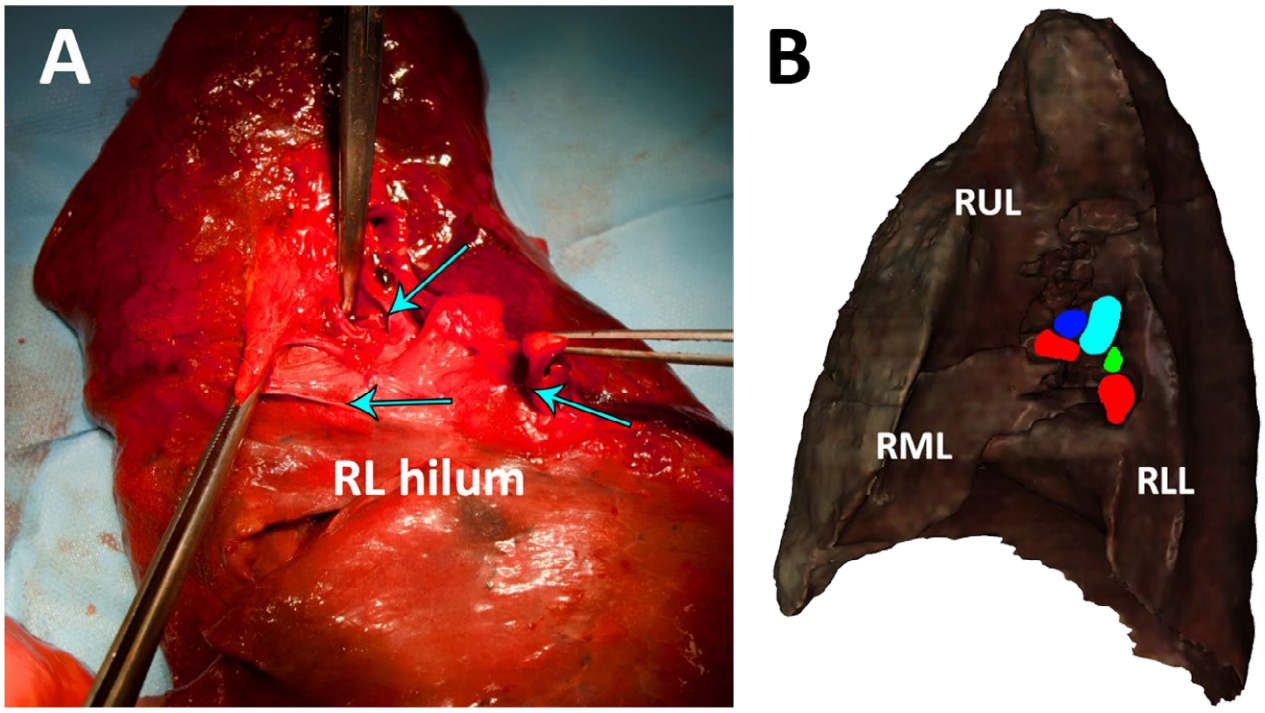

Figure 2. *The right lung hilum shows the pulmonary veins draining oxygenated blood into the left atrium.* (A) The anatomical variation observed in a 75-year-old female cadaver during undergraduate dissection classes. The arrows indicate the three pulmonary veins emerging from the hilum. (B) Representative image of the mediastinal surface of the right lung showing the putative area of the supernumerary pulmonary vein (green). RL=right lung; RUL=right upper lobe; RML=right middle lobe; RLL=right lower lobe. The image in panel B was adapted from "SECTRA Education Portal, Dissector Atlas v. 6.3.6, epsectra.com. Dissector Atlas is powered by Virtual Human Dissector and provided by Touch of Life Technologies".

such as in computed tomography (CT), angiography or magnetic resonance imaging (MRI). Failure to recognize supernumerary veins or abnormal drainage patterns may lead to misdiagnosis of conditions such as pulmonary embolism or to misinterpretation of vascular masses. Radiologists must be familiar with potential anatomical variations to ensure accurate diagnoses and avoid unnecessary interventions.

Advanced diagnostic imaging and cadaveric dissections offer clearer insights into such variations. Arrhythmias such as atrial fibrillation, can be effectively treated through radiofrequency ablation targeting ectopic foci. The success of this procedure relies on precise knowledge of normal pulmonary venous anatomy and its variations [5].

In conclusion, a comprehensive understanding of the anatomical features, including the number of pulmonary veins and left atrial ostia, is invaluable for clinicians. It facilitates accurate diagnosis and better planning and execution of surgical procedures, while reducing the risk of adverse events. As more cases of pulmonary vein variability come to light, clinicians must be well-equipped with this knowledge to optimize patient outcomes in diagnostic and therapeutic contexts.

AUTHOR CONTRIBUTIONS

Conceptualization: J.J.V.B., N.F., F.P.; Methodology: J.J.V.B., N.F., A.M.M., A.P., I.B.; Investigation: J.J.V.B., N.F., A.M.M., G.G., F.P.; Data curation: J.J.V.B., N.F., A.P.; Writing – original draft: J.J.V.B., N.F.; Writing – review & editing: I.B., A.M.M., G.G., A.P., F.P.; Visualization and figure supervision: I.B.; Supervision: F.P.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted in accordance with the Declaration of Helsinki.

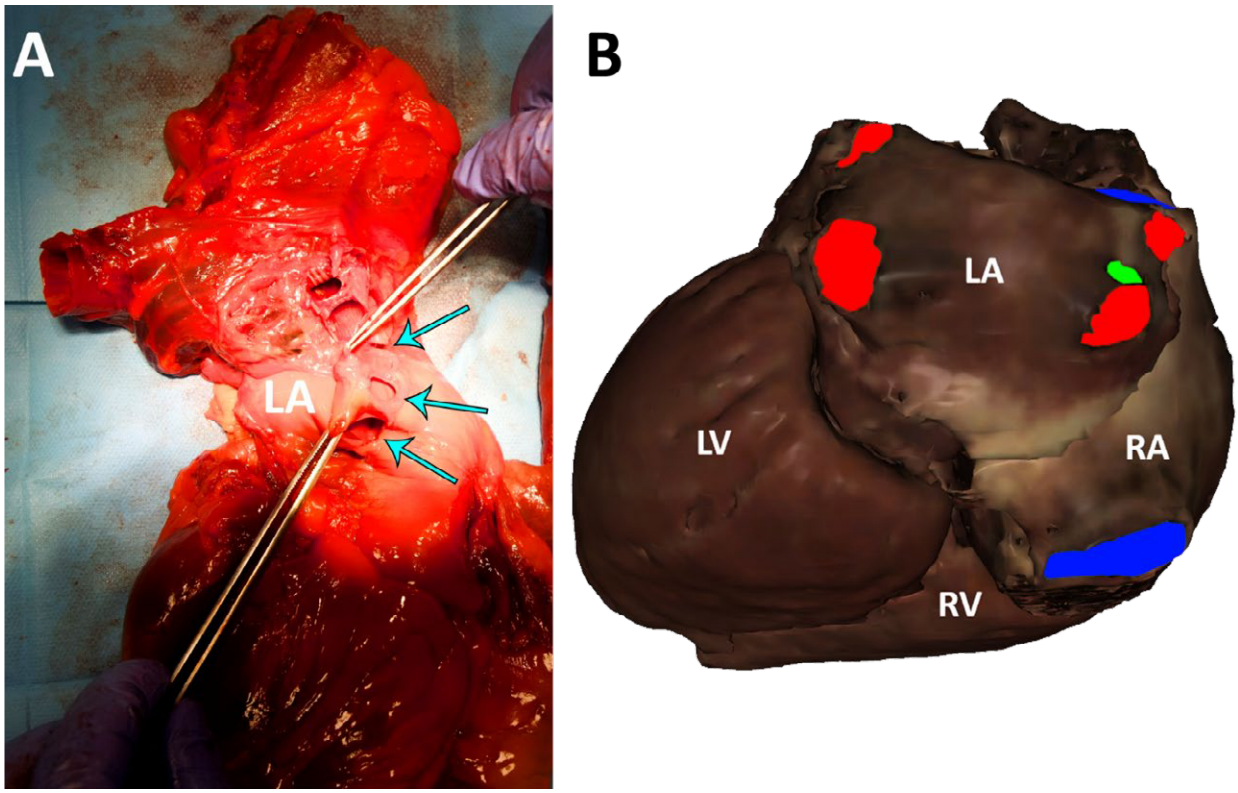

Figure 3. The left atrium shows three ostia for drainage of the right pulmonary veins. (A) The anatomical variation observed in a 75-year-old female cadaver during undergraduate dissection classes, showing the three ostia in the left atrium (arrows). (B) Representative image of the dorsal surface of the heart *in situ* showing the supernumerary ostium (green). LA=left atrium; RA=right atrium; LV=left ventricle; RV=right ventricle. The image in panel B was adapted from "SECTRA Education Portal, Dissector Atlas v. 6.3.6, epsectra.com. Dissector Atlas is powered by Virtual Human Dissector and provided by Touch of Life Technologies".

INFORMED CONSENT STATEMENT

Informed consent was obtained from the body donor prior to death for the use of donated tissues for educational and research purposes.

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Caffeine protects the blood brain barrier in type 2 diabetic rats, a histological and immunohistochemical study

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Abstract. Type 2 diabetes (T2D) is considered one of the metabolic conditions which may lead to degeneration of the central nervous system. The blood brain barrier (BBB) is recently recognized as a target that might be compromised in T2D. The pericyte and tight junction proteins, are major components of the BBB, that might be affected by diabetes. This may result in hyperpermeability of the BBB to toxic or hazardous substances, and this might affect its integrity. The current study addresses the effect of T2D on the BBB dysfunction and the role of caffeine to improve this condition. A rat model of T2D was used by feeding the rats with high calorie diet and injecting them with a single lower dose of streptozotocin. Diabetic rats were given caffeine orally for 5 weeks. At the end of the experiment (8 weeks), the rats were sacrificed, and their brains were processed for general histology and immunohistochemistry. The following markers were used: α-smooth muscle actin (α-SMA), occludin (tight junction protein) and transforming growth factor β1 (TGF-β1). There were degenerative changes in the cytoarchitecture of the CA1 area of the hippocampus. Immunohistochemistry revealed decrease in immunostaining of α-SMA and occludin and increase in TGF-β1 immunostaining, in diabetic rats. Caffeine intake resulted in improvement of the degenerative changes and reversal of the expression of the immunohistochemical markers. This study investigated the structural changes of the BBB in T2D, manifested by disruption of pericytes and tight junctions, with improvement of these findings after administration of caffeine.

Keywords: blood brain barrier, caffeine, occludin, pericyte, TGF-β1, Type 2 diabetes.

INTRODUCTION

Type 2 diabetes (T2D) is a common chronic condition that may lead to many health-related conditions (Garg and Duggal, 2022; Lenzi and Filardi, 2023). It may lead to several complications and is commonly associated

with changes in lifestyle such as having food with high calories as well as adopting a sedentary life (Basak and Laskar, 2024). Insulin resistance is worsened with the intake of high calorie diets (Han et al., 2023). The use of high calorie diets with streptozotocin (STZ) at a lower dose, is generally accepted to produce a model of T2D and insulin resistance in rodents (Zhang et al., 2021). There is strong correlation of T2D with various neurodegenerative diseases including diabetic encephalopathy (Szablewski, 2025). Additionally, several health impairment conditions can occur, like cognitive dysfunction, memory loss, and several structural abnormalities (Moran et al., 2019). The hippocampus is an important area of the brain which is linked to cognitive behavior, and it is well known to be influenced by metabolic impairment related to diabetes (Aderinto et al., 2023; Zhang et al., 2023). Rats that received high caloric diet, were reported to display disorganization of the structure of the hippocampus (Mezo-González, 2022). The local microvascular failure is a common underlying mechanism in diabetic complications. An important component of the microvascular structure is the pericyte, a cell present within CNS endothelial microvasculature; its dysfunction can cause microvascular diabetic complications (El-Ghazawi et al., 2024; Yu et al., 2025). Pericytes have important location in the CNS between neurons, endothelial cells, and astrocytes, and they are embedded within the basement membrane. They are crucial in controlling many important neurovascular functions; they participate in the BBB formation, vascular tone and remodeling (Chen et al., 2024). The transforming growth factor β (TGF-β) receptor signaling pathway modulates proliferation, differentiation, migration and attachment of pericytes and endothelial cells (Reyahi et al., 2015). However, during pathological conditions as diabetes, persistent elevation of TGF-β1 may result in dysregulation that promotes vascular remodeling, inflammatory response, and BBB dysfunction (Liu et al., 2021). The endothelial cells of the brain are specialized cells that make up one of the components of the BBB, characterized by high electric resistance, decreased transcytosis, and decreased paracellular permeability, which is mainly controlled by junctional molecules (Kadry et al., 2020). Tight junction is a type of these junctions that controls vascular paracellular transport by producing a strong electrical barrier. It is composed of some molecules as occludin, which may modulate paracellular vascular transport by making an electrical barrier that is highly resistant (Chen et al., 2024; Gopal et al., 2026). The BBB controls the homeostasis of the CNS by providing nutritional support and prevents the entry of neurotoxic substances from the circulation at the level of the microvascular endothelium (Rom et al., 2020). Caffeine is present in many food items and drinks, and it is well known for stimulation of the brain (Sharma et al., 2023) and has a high bioavailability and crosses lipid membranes quickly, therefore, it can quickly maintain blood levels (Song et al., 2024), and it can easily cross the BBB (Ikeda-Murakami et al., 2022). Caffeine has been suggested to ameliorate loss of memory associated with many neurodegenerative diseases (Ruggiero et al., 2022) and it is known for its anti-inflammatory and anti-oxidative properties (Ősz et al., 2022). Previously our lab reported an ameliorative role of caffeine on the brain of a rat model of type 2 diabetes, where it improved neurodegenerative changes of hippocampus and reduced inflammation (Othman et al., 2023). The aim of the present study is to use this animal model to investigate the disruption of pericytes, and tight junction protein of the brain, which might have a role in the integrity of the BBB in T2D. The possible role of caffeine as a therapeutic option is also addressed.

MATERIALS AND METHODS

Animals

The experiments were undertaken at the animal house of Arabian gulf university. Adult male Wistar rats of 250-300 g weight were used in the study. The rats were placed at 12/12 h light/dark cycle at room temperature of about 25 °C. They were allowed access to tap water. The experimental design and protocols were performed after obtaining approval from the committee of Animal Care and Use of College of Medicine, Arabian Gulf University, Bahrain, Ethics approval #: E006-PI-04/17.

Induction of diabetes and animal groups

Forty rats were used in this study, and the procedures performed were following our previously established model of T2D with modifications (Othman et al., 2023). Briefly, rats were classified in 4 groups: 10 rats per group. The control group (group 1): were rats that had low calorie diet (LCD) (SF 18-050, Specialty Feeds, Australia) and were injected with citrate buffer and had oral saline. The diabetic group (group 2): had high calorie diet (HCD) (SF-04-001, Specialty Feeds, Australia) and 4 weeks later, they received a single injection of Streptozotocin (STZ) (Sigma™, St. Louis, MO, USA) intraperitoneally at a dose of 35 mg/kg body weight (BW). The caffeine-treated diabetic group (group 3) received caf-

feine (Sigma TM, Saint Louis, MO, USA) orally through gavage at a dose of 100 mg/kg BW, for 5 weeks every day. Caffeine administration began at the start of week 4 (one week prior to STZ injection) and continued until the end of the study (week 8). Caffeine only group (group 4) were nondiabetic rats which had caffeine and LCD. By the end of the study (8 weeks), the animals were sacrificed, after inhalation of CO2, the brains were removed and put in formalin fixative and then processed for general histology and immunohistochemistry.

Measurements of blood glucose level

Evaluation of blood glucose levels was done to all rats at the start of the study (day 1), after 72 hours of STZ injection (the middle of week 4), and at the end of the study (week 8). A blood sample was withdrawn from distal ends of rat tails, after 12 h fasting, using a device for measurements of blood glucose (Accu-Check Active, Roche Diagnostics, Mannheim, Germany).

Histological evaluation

The brains were removed, from all groups, and placed in 10% neutral buffered formalin fixative. The area that contains the hippocampus was identified between 3-6 mm posterior to bregma (Paxinos and Watson, 2013). After processing, coronal sections were obtained from the paraffin blocks at 5 μm, and then processed for staining with hematoxylin and eosin (H&E) for general morphological evaluation. Photographs were obtained using 40X objective lens with emphasis to CA1 areas containing blood capillaries.

Immunohistochemistry

Five μm coronal sections, were processed for immunohistochemistry using (Vectastain Elite ABC Kit Universal, Vector laboratories, UK) according to the instructions of the manufacturer. The sections were incubated overnight in the refrigerator (4 °C) with the following antibodies, separately: anti-alpha-smooth muscle actin (α-SMA), a marker for pericytes, mouse monoclonal antibody, ab7817, Abcam, UK, anti-occludin (tight junction marker), ab216327, a rabbit monoclonal antibody, Abcam, UK, and anti-transforming growth factor-beta 1 (TGF-β1), ab215715, a rabbit monoclonal antibody, Abcam, UK. Visualization of the reaction was done using DAB (3,3-diaminobenzidine tetrahydrochloride, and counterstaining of the sections was performed by Harris hematoxylin. Slides were viewed and photographs captured by a light microscope with a digital camera (Axio Scope A1, Carl Zeiss, Microscopy, Germany). Immunohistochemistry photographs were analyzed quantitatively for percent area using ImageJ® software (Wayne Rasband NIH, Bethesda, MD, USA). Six sections were used for each animal and 6 non-overlapping fields per section were examined. All photographs were obtained using the objective lens 40X. All histological and immunohistochemistry procedures were performed according to (Bancroft et al., 2019).

Statistical analysis

Statistical evaluation was done by the software Statistical Package for Social Sciences (SPSS) version 27 for Windows (IBM Corp., Armonk, NY, USA). The variables were expressed as mean ± standard deviation (SD). Data were analyzed using one way analysis of variance (ANOVA) and Tukey post hoc test. P values < 0.05 are considered statistically significant.

RESULTS

1. Blood glucose level

At day 1, blood glucose levels were within normal range with no statistically significant difference among the groups (ANOVA; F=0.668, P= 0.578). At week 4, significant difference was detected between the groups (ANOVA: F=121.87, P<0.0001). Group 2 demonstrated markedly elevated blood glucose levels compared with the other groups, while group 3 showed mildly increased levels. Groups 1 and 4 maintained relatively stable glucose levels. At week 8, significant intergroup differences persisted (ANOVA: F = 149, P <0.0001). Group 2 continued to exhibit the highest blood glucose levels, followed by group 3, which exhibited near normal values, whereas group 1 and 4 remained with comparable ranges (Figure 1).

2. Histological study

Evaluation of H&E sections of the CA1 (Cornu Ammonis 1) of the hippocampal region of the control group displayed normal structure with small pyramidal neurons that exhibited pale cytoplasm and nucleus. Blood capillaries appeared intact, together with other glial cells. The cells of the diabetic group appeared with dark cytoplasm and nucleus, and the blood capillaries looked disorganized. After caffeine administration, there

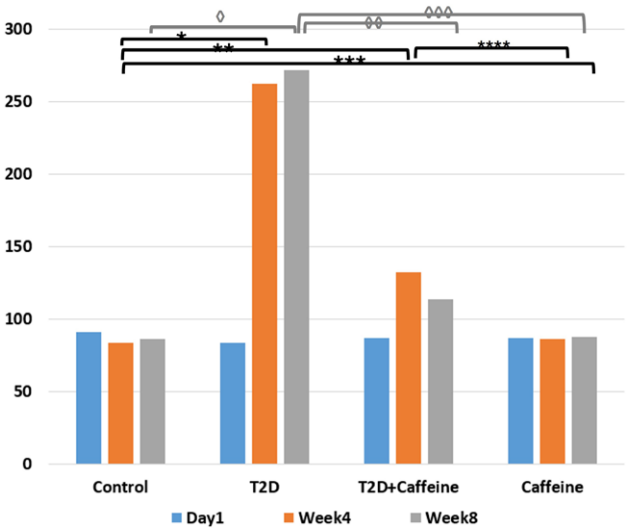

Figure 1. Bar graphs showing blood sugar levels among the tested groups at day 1, week 4 and week 8. Tukey post-hoc test showed that the significant difference in week 4 was among the control and T2D group *(P=0.0004), control vs T2D+caffeine **(P<0.001), T2D vs caffeine ***(P<0.0001) and between T2D vs caffeine groups ****(P=0.0008). At week 8 the significant difference was among the control group vs T2D ◊(P<0.0001), T2D group vs the T2D+caffeine groups ◊◊(P<0.0001) and between T2D group vs the caffeine group ◊◊◊(P<0.0001).

was improvement of many cells and capillaries appeared intact. The histological sections of rats that received only caffeine, which serves as a control as well, revealed results similar to the control (Figure 2).

3. Immunohistochemistry

3.a. Caffeine reversed α SMA downregulation

The α SMA immunohistochemistry of CA1area of the hippocampus of T2D rats showed reduction of immunoreactivity of the pericytes in the blood vessels, compared to the controls. Diabetic animals which were administered caffeine displayed upregulation of α SMA expression (Figure 3).

3.b. Caffeine reversed occludin downregulation

The occludin immunohistochemistry, which is a marker of tight junction, of CA1area of the hippocampus of T2D animals showed reduction of immunoexpression, compared to the controls. Diabetic animals which were administered caffeine displayed an increase of occludin immunoreactivity (Figure 4).

3.c. Caffeine normalized TGF-β1 upregulation

For the TGF-β1 immunohistochemistry, CA1area of the hippocampus of diabetic rats showed marked immunoreaction, compared to the controls. Diabetic rats that received caffeine revealed decrease of TGF-β1 immunoreactivity (Figure 5).

3.d. Quantitative analysis of immunohistochemical marker expression

Quantitative evaluation of the percent area of αSMA and occludin immunoreactivity showed a significant decrease in the immunoreaction in diabetic rats in relation to the controls, on the other hand, caffeine pretreatment to diabetic rats increased their reaction. For TGF-β1 expression, there was an increase in the reactivity in T2D animals in relation to the control, meanwhile caffeine administration to diabetic rats resulted in downregulation of its expression (Table 1).

DISCUSSION

Type 2 diabetes is becoming a serious metabolic disease with potential disabling complications. The pathophysiology of the disease is complicated and multifactorial that makes management a real challenge. In this study we are reporting the effects of caffeine as a treatment modality in a rat model of T2D by investigating the integrity of the BBB. Diabetes is a metabolic disease characterized by chronic hyperglycemia which may lead to injury to vital organs due to damage to microvasculature, which might affect neuronal function, and white matter disruption (Rom et al., 2020; Zhou et al., 2023). It was reported in the literature that the breakdown of BBB is linked to diabetes progression and the exact mechanisms for BBB permeability and therapeutic approaches, is not currently known (Rom et al., 2020). In this study we found that T2D disrupts the integrity of BBB through affecting pericytes and tight junction proteins. Staining with H & E detected degeneration of hippocampal neurons in diabetic rats, which might be related to BBB leakage and neurotoxic or vascular substances, which may exaggerate neuronal destruction (Lin et al., 2021). Leakage of BBB was evidenced in diabetic animal models as well as in human studies (Liu et al., 2021; Meijer and Gorter, 2024). The current study found downregulation of the αSMA, which is a marker of pericytes, in diabetic rats, a finding that signifies loss of the contractile ability of pericytes and pericyte dysfunction (Liu et al., 2021). The α-SMA was chosen as a marker for the

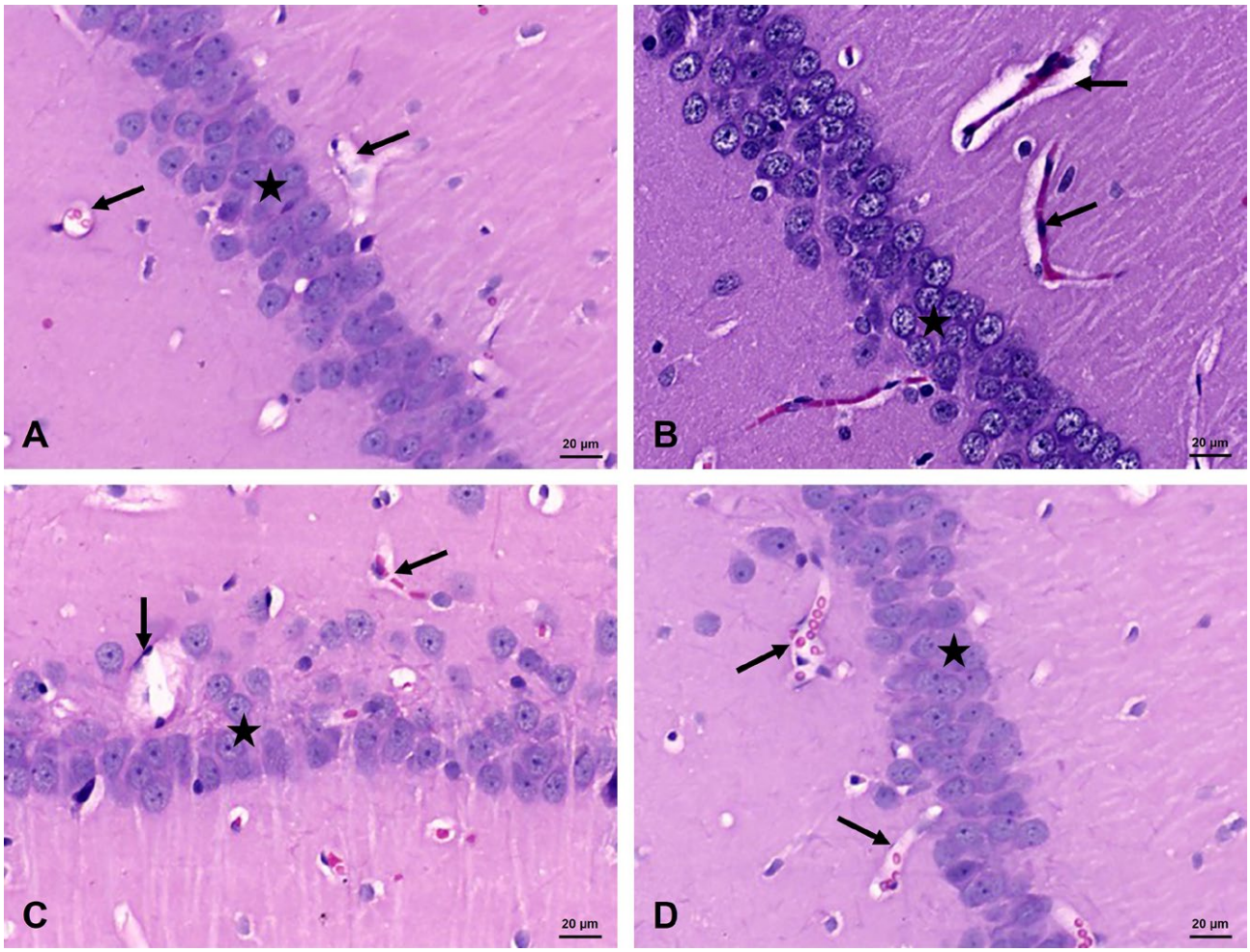

Figure 2. Photomicrographs of the CA1 area of the hippocampus stained with hematoxylin and eosin (H&E). (A) Control group showing lightly stained pyramidal neurons with open vesicular nuclei, intact blood capillaries, and glial cells, consistent with normal neuronal morphology. (B) Diabetic group revealing darkly stained neurons with condensed pyknotic nuclei, consistent with neuronal degeneration, and congested, disrupted blood capillaries. (C) Caffeine-treated diabetic group showing partial recovery with predominantly lightly stained neurons and intact blood capillaries. (D) Caffeine-only group displaying neuronal morphology and capillary integrity comparable to the control group. Arrows, blood capillaries; asterisks, pyramidal cells.

pericytes, while it is not specific, and it stains other cell types and this presents a limitation to our study. In figure 3 we are illustrating the blood capillary with a position more suitable for pericytes. The pericytes can be stained with more specific markers, and this will be investigated in detail in our future studies. Diabetes is known to increase the risk of dementia and neurodegeneration, but the effects of hyperglycemia on pericyte/BBB function is controversial. Some studies proposed that the dysfunction of pericytes and BBB affection is related to oxidative stress and reduced ATP production resulting from prolonged hyperglycemia (Liu et al., 2021). Under physiological conditions, the pericytes are crucial for the formation of the BBB, regulation of neurovascular system, inflammatory response, and removal of toxic materials from the brain (Lin et al., 2021). In the current study, in diabetic rats, there was a decline in the occludin expression levels, which is a tight junction protein, suggesting impaired BBB as previously reported in mice, where they found degradation of TJ proteins, which can suggest it to be a mediator of BBB dysfunction (Machida et al., 2017). Immunohistochemical evaluation, in this study, revealed increased expression of TGF-β1 in diabetic rats, compared to controls, which decreased after caffeine administration. Physiological TGF-β is important for BBB maintenance, through regulation of endothelial-pericyte interactions. The increased TGF-β1 expression in diabetic animals may represent a compensatory pro-

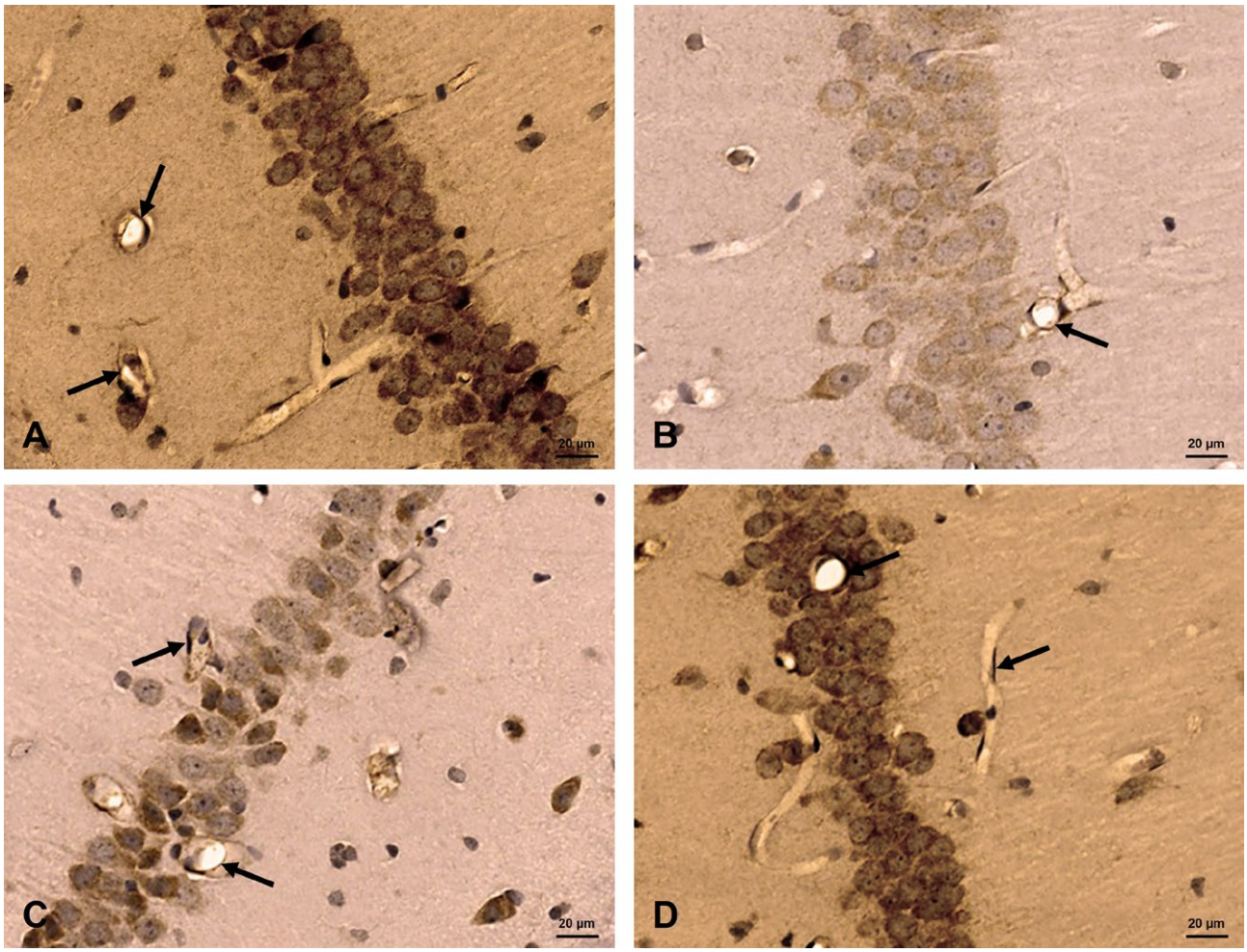

Figure 3. Photomicrographs of the CA1 area of hippocampus immunostained with anti-α SMA. (A) Control group showing strong diffuse brown immunoreactivity and the elongated pericyte morphology wrapping around capillaries. (B) Diabetic group shows that staining intensity is reduced and fewer stained pericyte profiles are visible and this is consistent with pericyte loss in the diabetic state. (C) Caffeine-treated diabetic group reveals partial recovery with staining intensity appearing intermediate between panel A and B. (D) Caffeine only treated sections display staining intensity comparable to panel A, which signifies that caffeine alone does not affect the integrity of pericytes. Arrows, α SMA positive pericyte associated with blood capillaries.

tective mechanism, which may become maladaptive when chronically activated (Li et al., 2022). In this context, the caffeine induced reduction of TGF-β1 detected in this study may indicate normalization of the TGF-β1 overactivation rather than inhibition of its protective functions. Pericytes release certain factors, to improve the integrity of the tight junctions and enhance the stability of the BBB as TGF-β, and through this TGF-β signaling pathway, pericytes activate tight junction protein expression as occludin and others, which are important for formation of a high resistant barrier (Chaulagain et al., 2023). The release of TGF-β might result from thickening of the basement membrane, which may activate fibronectin release by pericytes (Li et al., 2022). In the absence of pericytes, endothelial cells display increased permeability allowing dysregulated paracellular transport and jeopardize the function of BBB (Kim et al., 2025). Previously, the expression of these proteins was detected in another study, in diabetic rats, which they may be associated with pericyte dysfunction, and loss of tight junction integrity of endothelial cells (Fan et al., 2015). In the current study caffeine was administered to the diabetic animals, which led to improvement of the BBB parameters tested and structure of hippocampal neurons. When caffeine is consumed on a regular basis, it enhances adaptive mechanisms in the hippocampus, which may affect the primary metabolism and modulate synaptic plasticity (Lopes et al., 2023). Additionally, this role of caffeine

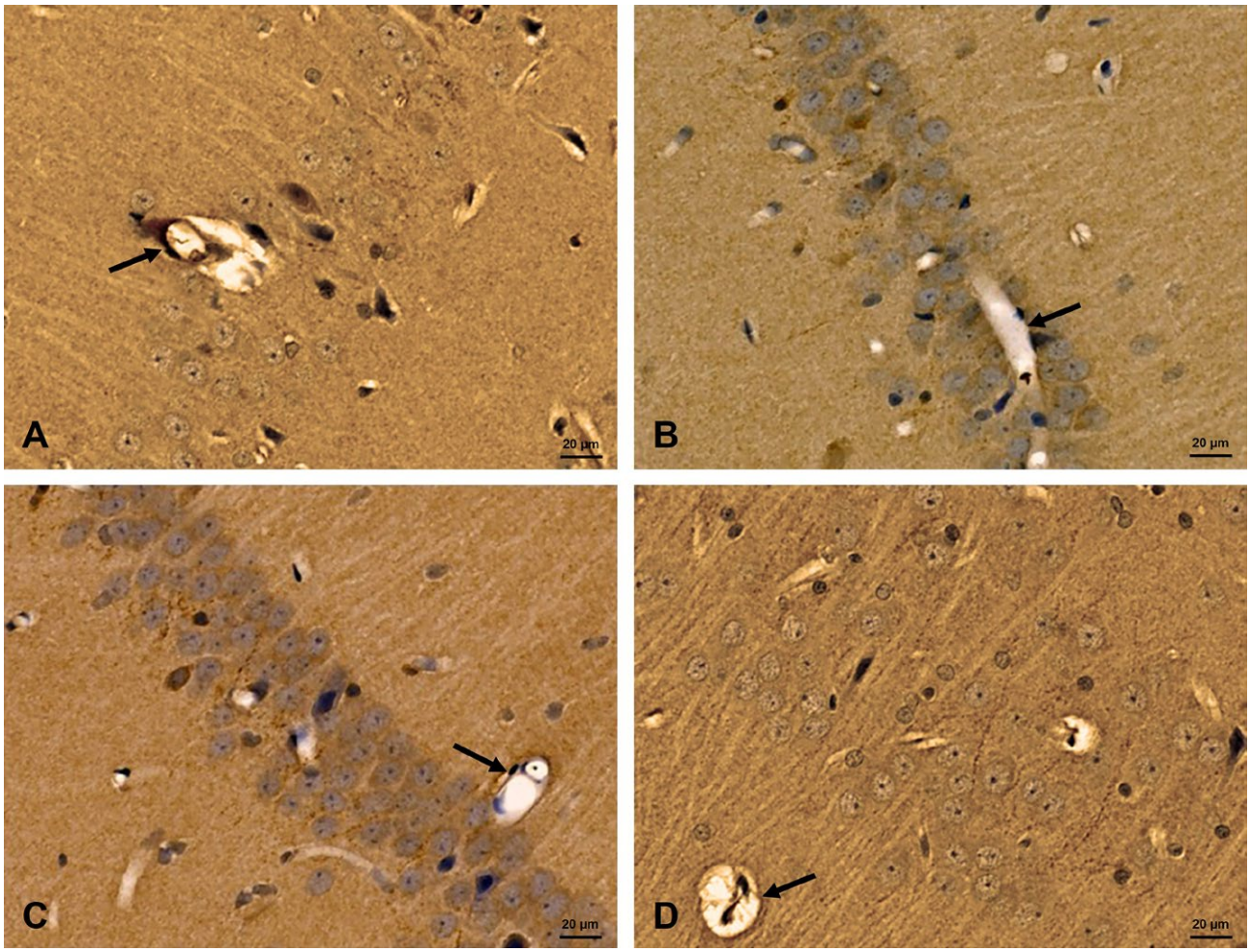

Figure 4. Photomicrographs of the CA1 area of hippocampus immunostained with anti-Occludin. (A) Control group reveals the strong brown immunoreactivity of the tight junction component. (B) Diabetic group shows a marked decrease in staining intensity of the tight junctions. (C) Caffeine-treated diabetic group reveals a partial recovery with staining intensity intermediate between A and B. (D) Caffeine only treated animals shows the staining of occludin, similar to control. Arrows, blood capillaries.

in neuroprotection might be due to anti-inflammatory and glial cell modulation (da Silva Neves et al., 2025). This study is focusing on the role played by caffeine to enhance BBB function, by reversing pericyte loss and enhancing tight junction protein efficiency. Caffeine can provide neuroprotection which is related to its ability to block adenosine receptors (Lopes et al., 2023). Caffeine has both hydrophilic and lipophilic nature, it can cross biological membranes, including BBB, thus having stimulatory effects in the central nervous system. Additionally, caffeine has anti-inflammatory, and antioxidative effects and neuroprotective effects of caffeine were reported by previous studies in some neurodegenerative conditions and stroke (Sharma et al., 2025). A meta-analysis in 2020 observed that regular intake of caffeine revealed a lowered risk of Parkinson disease and its progression (Hong et al., 2020). The relation between caffeine intake and the risk of dementia and AD was also investigated in another meta-analysis in 2024; they detected lowered risk of dementia and AD, with caffeine intake (Li et al., 2024). Caffeine has been shown to have a significant role in protecting against damage of the brain such as neurodegeneration, and cognitive impairment. Caffeine has a role in blocking oxidative stress and controlling inflammatory pathways in brain injuries. The anti-inflammatory and antioxidant roles are not through blocking of adenosine receptors, indicating alternative pathways (Vargas-Pozada et al., 2022; Awashra et al., 2026). A role for activated microglia and release of certain cytokines such as IL-6 and TNF-α, can mediate the neurotoxicity. These anti-inflammatory effects of caffeine can be the mechanism of the neuroprotection via minimizing damage to

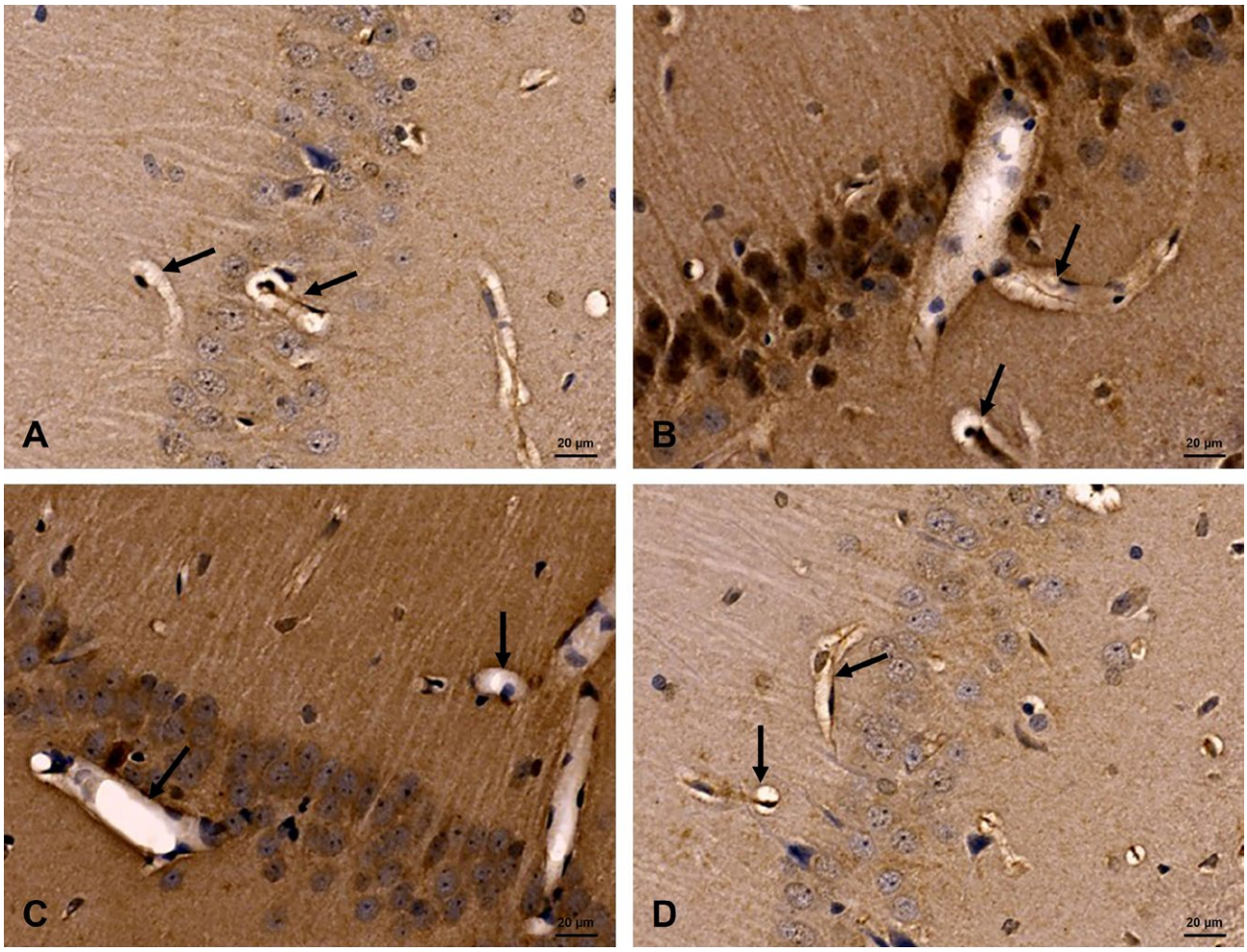

Figure 5. Photomicrographs of the CA1 area of hippocampus immunostained with anti-TGF-β1. (A) Control group shows mild immunoreactivity of cells. (B) Diabetic group shows increase in staining and strong diffuse brown immunoreactivity. (C) Caffeine-treated diabetic group reveals a partial decrease in immunoreactivity. (D) Caffeine only treated animals shows minimal staining similar to control. Arrows, blood capillaries.

Table 1. Immunoreactivity of different markers in the studied groups.

	α SMA positive area Mean ± SD	Occludin positive area Mean ± SD	TFG β1 positive area Mean ± SD
Control	11.05±0.9	14.36±0.831	7.17±0.7
D.M	3.04 [a,c,d] ±0.557	6.25 [a,c,d] ±1.1	15.5 [a,c,d] ±1.04
D.M + Caffeine	7.73 [a,b,d] ±0.775	10.45 [a,b,d] ±1.1	9.93 [a,b,d] ±0.973
Caffeine	10.3 [b,c] ±0.654	13.43 [b,c] ±1.2	6.93 [b,d] ±1.401

Standard deviation (SD), one way analysis of variance (ANOVA) was used followed by *Tukey post hoc* test for multiple comparisons [a] significant compared to control group, [b] significant compared to DM group, [c] significant compared to DM treated with Caffein group, [d] significant compared to caffein only treated group.

tissues and improving survival of neurons (Othman et al., 2023). An in vivo and invitro study, investigating the protective role of caffeine in lipopolysaccharide-induced injury to retinal cells; they found decrease in inflammatory expression of IL6, TNFα, IL-β1. Additionally, caffeine upregulated the lowered tight junction protein (ZO-1) expression (Conti et al., 2022). This aligns nicely with the findings in this study regarding the effect of caffeine in enhancing BBB integrity and occludin upregulation. It was reported in the literature that the breakdown of the BBB is one of the mechanisms of dementia and Alzheimer's disease (AD) (Rom et al., 2020; Kim et al., 2025). The effects of caffeine in protecting against AD can be due to blocking of adenosine receptors, which are important in the formation of amyloid beta, which mediates neurotoxicity in neuronal culture models of AD (Stock-

well et al., 2017). Caffeine can also be considered a neuroprotective drug in cases of PD for delaying the progress of the disease, which is mediated by blocking adenosine receptors, that suppresses dopamine 2 receptor, and via antioxidant effect (reviewed in: Song et al., 2024).

LIMITATIONS OF THE STUDY

"A limitation of the current study is the use of relatively high caffeine dose (100 mg/kg body weight). This dose was chosen based on our previous studies using the same diabetic rat model, in which the same dose revealed neuroprotective effects to the brain by ameliorating inflammation and gliosis (Othman et al., 2023). Additionally, a study from other researchers, used several doses of caffeine (10-120 mg/kg body weight), in a mouse model of sleep deprivation (Onaolapo et al., 2015). Although this dose exceeds typical human caffeine consumption values, there is probably a species-specific difference in metabolism and pharmacokinetics, which may make this dose achieve pharmacological effects in the animal model, rather than mimic typical human dietary consumption. Future studies using lower doses of caffeine are warranted". This study did not include electron microscopic evaluation, which could have addressed detailed structure of the BBB. Additionally, behavioral testing was not performed, which might have highlighted the cognitive function. These studies are among our future plans to have more insights about the BBB, and the functional impact of its compromise.

CONCLUSION

The current study showed structural changes of different components of the BBB in the CA1 area of hippocampus in a rat model of T2D. Additionally, it highlighted the ameliorative effects of caffeine against these changes that could be related to its ability to enhance the BBB. Future studies are in progress to investigate the role of caffeine in such protective roles.

AUTHOR CONTRIBUTIONS

Conceptualization: MA Othman, YI Tayem. Laboratory work: A Rashid, N Bakhit. Manuscript drafting: MA Othman, N Bakhit. Data analysis: A Rashid, N Bakhit. Critical review of the manuscript: MA. Othman, YI Tayem. Final manuscript approval: All authors.

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Variations in the dimensions of the sacrum and its implications for Forensic Anthropology

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Abstract. With rising numbers of unidentified bodies in South Africa, the South African Police Service (SAPS) requires effective skeletal identification techniques. While the skull and pelvis are highly dimorphic, taphonomic fragmentation often renders them unavailable at forensic scenes. The sacrum is frequently well-preserved and exhibits population specific sexual dimorphism. This study aimed to evaluate the dimensions and three-dimensional (3D) shape of the ventral sacrum in a contemporary South African sample. Retrospective Computer Tomography (CT) scans of 51 black South African adults (26 females, 25 males) were obtained from Steve Biko Academic hospital. Utilising MeVisLab © (v.3.0.2), the sacra were isolated via threshold-based segmentation, and 3D landmarks were placed on the ventral sacral surface. Linear dimensions were calculated and evaluated using descriptive statistics and discriminant function analysis (DFA), while shape variations were assessed using a multivariate analysis and a two-group permutation test. Black South African males exhibited a significantly greater sacral height than females (p=0.004). The permutation test confirmed significant sex-based variation in overall ventral sacral shape (p= 0.005). DFA models predicted sex with a moderate 72.55% accuracy based on linear dimensions, but achieved an exceptional 94.12% accuracy utilising 3D shape coordinates. ROC analysis substantiated the predictive value of the ventral linear metrics (AUC = 0.73; 95% CI: 0.60 – 0.85; p = 0.004) for sacral height, yielding an optimal cut-off value of 103.41 mm (Sensitivity: 76 %, Specificity: 65.38 %). Sexual dimorphism significantly influences both the metrics and morphology of the ventral sacrum of black South Africans. Due to these distinct variations, the ventral sacral surface, particularly its 3D shape, provides a highly reliable alternative for accurate forensic sex estimation.

Keywords: sacrum, anatomical variation, biological profile, geometric morphometrics, Sexual dimorphism.

INTRODUCTION

With rising incidences of unsolved forensic cases, the South African Police Service (SAPS) needs reliable methods to determine the sex and population affinity of unknown victims. Forensic anthropologists assist the SAPS in their investigation by creating a biological profile using skeletal remains (L'Abbé and Steyn, 2012). The biological profile includes the sex, population

affinity, age, stature and possible cause of death of the deceased (Krüger et al, 2018; Spradley, 2016).

Due to biological and geographical taphonomy, determining the biological profile of a victim may be challenging (Klales et al.,2009; Iscan and Steyn, 2013). Creating a biological profile for the deceased has proven to be a difficult task worldwide, as forensic anthropologists rarely receive a full skeleton from the police (Krüger et al, 2018). Often, the skull and limbs of the individual may not be present due to the harsh environmental conditions, scavenger activity or the nature of the crime (Krüger et al, 2018; Brits et al, 2020). This poses a challenge to investigators and forensic anthropologists alike, in trying to determine the sex, ancestry and age of the unknown individual in the attempt to solve a crime (Krogman and Iscan 1986). Due to these challenges, forensic anthropologists need to find additional, effective techniques to create reliable biological profiles of the deceased.

Numerous books and articles have been published based on metric and non-metric data of various bones of the human skeleton to assist forensic anthropologists in determining the sex and population affinity of the deceased (Flander, 1978, Iscan and Steyn 2013; Rusk and Ousley, 2016).

The sacrum is an imperative bone which can be useful in determining the ancestry and sex of an unknown victim, as it is often preserved well after death (Flander,1978, Rusk and Ousley, 2016). Due to this fact, the sacrum has captured the attention of forensic anthropologists in their quest to find reliable methods to determine the identity of the deceased (Flander, 1978; Rusk and Ousley, 2016).

Geometric morphometric techniques have been used to classify the human sacral landmarks based on their three dimensional shape (Passalacqua et al, 2010). Vollner and colleagues (2011) cross-validated sex and ancestry-sex classification accuracy measurements in a study performed on black and white Americans, which yielded an 89% and 65.6% accuracy for sex determination rate on the os coxae and sacrum respectively. Sexual dimorphism seems to be population specific as expected and therefore, population specific guidelines are needed (Iscan and Steyn, 2013).

ANATOMY OF THE SACRUM

The sacral bone is a large triangular bone situated at the base of the vertebral column where it contributes to the formation of the pelvis (Bogduk, 2005). The sacrum consists of five fused sacral vertebrae (Drake et al ,2009). It has a broad thick upper end, which articulates with L, and it then tapers to a blunt tip inferiorly where it articulates with the coccyx (Drake et al, 2009; Bogduk, 2005).

The sacrum has a smooth ventral surface (Fig 1A) that is concave and a rough dorsal surface which is convex, with both surfaces containing sacral foramina (Drake et al, 2009; Bogduk, 2005). The sacral promontory, which forms part of the pelvic inlet, is located on the ventral surface of the first sacral vertebra (Drake et al, 2009). The ala of the sacrum is located on its lateral surface, which articulates to the ilium of the os coxa to form the sacroiliac joint (Drake et al, 2009).

The ventral surface of the sacrum is of particular importance as it forms part of the birth canal, which is known to be sexually dimorphic and population specific (Betti, L., 2014).

CONGENITAL CONDITIONS ASSOCIATED WITH THE SACRUM

Forensic anthropologists must be aware of congenital abnormalities of the skeleton when examining skeletal remains. Sacral defects commonly occur in three observed patterns: the absence of the vertebrae (for instance sacral agenesis), hemivertebrae (hemisacral), and defects of the neutral arch which results in sacral spina bifida (Stanley et al, 1979). A common congenital abnormality observed in the sacral region, includes the lumbosacral transitional vertebrae (Apazidis et al, 2011). The shape and size of the sacrum is affected by the presence of a lumbosacral transitional vertebra (LSTV), noted at the L5-S1 junction (Apazidis et al, 2011).

Lumbosacral transitional vertebra is associated with varied degrees of geometrical changes in different anatomical features of the human sacrum (Mahato, 2016). The LSTV is the most common congenital abnormality, such a transitional vertebra may present with abnormal variations in the normal lumbosacral articulations between the transverse process of the inferior lumbar vertebra and the sacrum (Mahato, 2016). When the L5 vertebra is fused to the sacrum it is referred to as sacralisation of L5, which results in the presence of only four lumbar vertebrae. When S1 is separated from the sacrum is referred to as lumbarisation of S1 (Connolly et al, 2003, Mahato, 2016).

SEXUAL DIMORPHISM OF THE SACRUM

Anatomical textbooks commonly describe the sacrum as “long and narrow” in males and “short and broad” in females (Benazzi et al.,2009; Iscan and Steyn

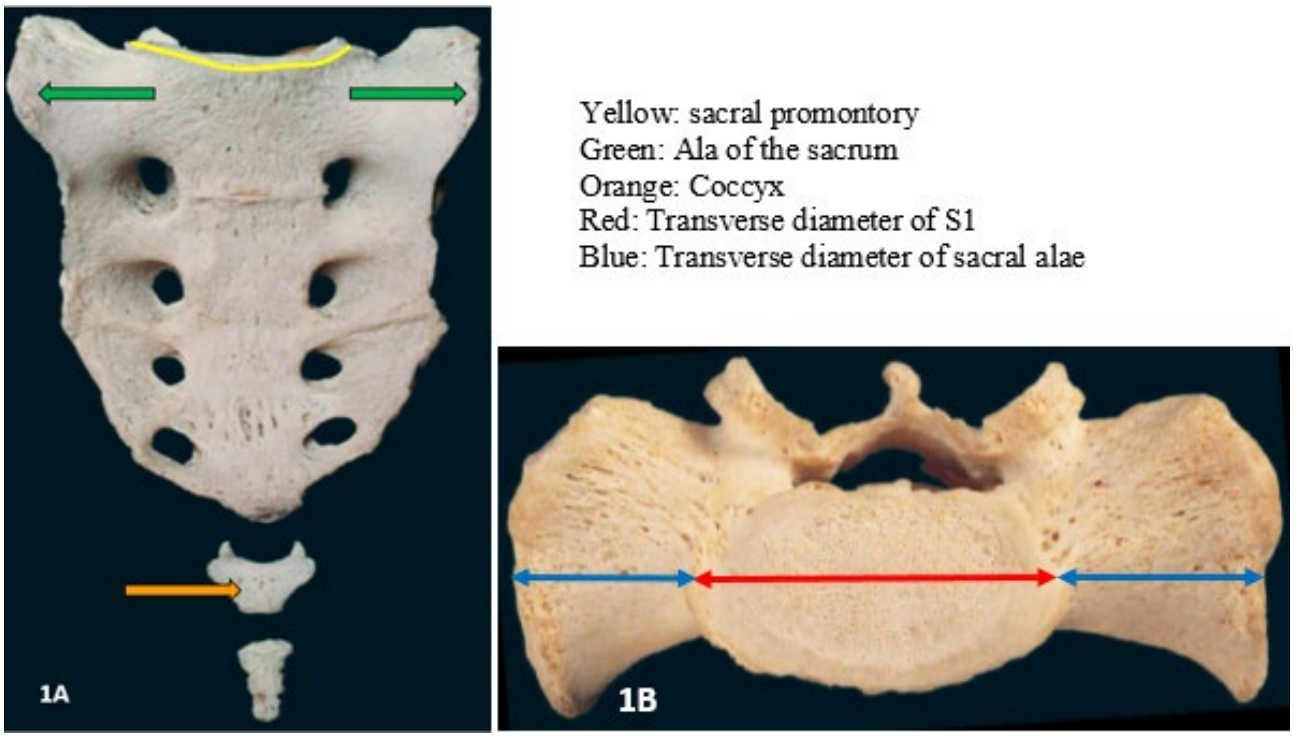

Figure 1. A: Ventral and B: superior surface of the sacrum indicating bony landmarks Abraham et al (2008).

2013; Moore and Dalley, 2018). The sacrum is accepted to be sexually dimorphic due to its effect on the morphology of the shape of the pelvic inlet and outlet. The female sacrum is less curved, resulting in a larger, more oval shaped pelvic outlet (Flander, 1978). The discrete differences in the size, curvature, number of sacral vertebrae, extent of the articulation surfaces and the ratio of the first sacral vertebra to alae (S1/alae ratio) which is the ratio between the width of S1 and the width of the sacrum and the ratio between the width of the alae and the width of the sacrum, it plays a critical role in determining sex based on the sacrum (Iscan and Steyn, 2013)

With regard to the dimensions of the sacrum, it has been demonstrated to be an accurate indicator of sex differences using sacral breadth measurements (Trotter, 1926). According to Flander (1978), the anteroposterior dimensions and transverse length of the first sacral vertebral body are highly reliable indicators of sex (Flander,1978; Rusk and Ousley, 2016). In addition to these sexually dimorphic diameters, the S1/alae ratio, also referred to as the base-wing index, is an accurate metric for sex estimation (Kimura, 1982; Rusk and Ousley 2016).

A study performed by Rusk and Ousley (2016) used shape analysis based on multiple landmarks placed on the sacrum to determine variations among sexes between white and black Americans. They noted the sacrum had a classification accuracy ranging between 60.2 – 98.0% for sex determination and 60.0 – 95.8% for population affinity estimation.

POPULATION / ANCESTRAL VARIATIONS

Steyn and collegues (2016) explained the distinct morphology of South Africans' bones due to the population originating from various locations with distinct climatic conditions. Tague (2007) discovered that white Americans have a larger sacrum as compared to that of black African-Americans. Furthermore, distinct sexual dimorphism exists across planes: males exhibited a greater S1 width in both the anteroposterior and transverse planes, whereas females displayed longer S1 transverse processes alongside larger transverse diameters and circumferences of the pelvic inlet (Tague, 2007). In contrast, Rusk and Ousley (2016) determined that the dorso-ventral curvature of the sacrum is more accurate in estimating population affinity (White vs Back Americans) than for sex estimation, despite the traditional reliance on this curve for sex determination.

Given the high preservation rate of the sacrum in skeletonised remains and the documented sexual dimorphism across diverse populations, this study aimed to

evaluate variations in the dimensions and shape of the ventral surface of the sacrum within a black South African sample.

MATERIAL AND METHODS

Material

A quantitative descriptive study was performed using Computed Tomography (CT) scans of 51 black South African adults (26 females, 25 males). Retrospective CT scans were selected from a repository at the Radiology Department of an academic hospital in Pretoria. Ethical approval (SMUREC/M/158/2022: PG) was obtained the research ethics committee at Sefako Makgatho Health Sciences University prior to data collection.

Patients were scanned in the supine position from the head to mid-thigh. slice thickness ranged between 2 to 2.5 mm. CT scanned data was collected in DICOM (Digital Imaging and Communications in Medicine) format which was imported into the fee medical image processing software, MeVisLab © v.3.0.2.

Methods

The collected DICOM files were imported into the MeVisLab © v.3.0.2 to visualise the skeletal structures of the articulated pelvis through threshold-based segmentation. Segmentation is an automated process that identifies the best limit between grey values of different densities, resulting in either a soft-tissue or hard-tissue 3D rendering (Spoor et al., 2000). For these CT scans, the attenuated threshold values typically ranged between 1100-1200 Hounsfield Units (HU) for bone tissue and 400 – 500 HU for soft tissue.

Ten landmarks were selected from literature to determine the dimensions and the shape of the sacrum (Table 1, Figs. 2 and 3). Table 2 defines the specific measurements calculated from the landmarks placed on the 3D sacral reconstruction.

By placing the selected landmarks on the 3D ventral surface of the segmented sacrum, the landmarks were exported in XML format as x, y and z coordinates. The data was then imported into MS Excel to calculate the linear distances listed in Table 2. Normality testing and basic descriptive statistics were performed using the PAST v4.11 programme to determine the average dimensions of the ventral surface of the sacrum in black South African males and females. A Shapiro Wilk test were performed to evaluate data distribution and to select the appropriate statistical tests to assess significant difference between sexes.

To evaluate the effectiveness and discriminative ability of the sacral measurements in distinguishing between male and female individuals, a Receiver Operating Characteristic (ROC) curve analysis was conducted. The Area Under the Curve (AUC) was calculated for each measurement to evaluate its overall diagnostic accuracy, where an AUC of 0.5 indicates random guessing and $0.7 \leq 0.9$ indicates perfect discrimination, only applied to statistically significant ($p \leq 0.05$) values. The optimal cut-off values maximising both sensitivity and specificity were determined. This ROC analysis protocol followed the methodology described by D'Arcangelo et al. (2019).

RESULTS

The results of the Shapiro-Wilk test indicated that the S1/ala ratio was non-parametric in females, while the width of the ala was non-parametric in males. Independ-

Table 1. Landmarks to be placed on the ventral surface of the sacrum.

Landmark	Abbrev.	Definition
Sacral promontory	SPM	The most protruding point in the midline along the superior margin of S1 (referred to as the sacral promontory)
Right lateral S1	RLS1	The most lateral point along the right superior margin of the first sacral vertebra
Left lateral S1	LLS1	The most lateral point along the left superior margin of the first sacral vertebra
Right lateral ala	RLA	The most lateral point along the superior margin of the right ala
Left lateral ala	LLA	The most lateral point along the superior margin of the left ala
Sacral apex	SA	The tip of the sacrum, on the inferior margin of S5
Ventral S2	VS2	The most protruding point along the antero-superior margin S2 in the midline
Ventral S3	VS3	The most protruding point along the antero-superior margin S3 in the midline
Ventral S4	VS4	The most protruding point along the antero-superior margin S4 in the midline
Ventral S5	VS5	The most protruding point along the antero-superior margin S5 in the midline

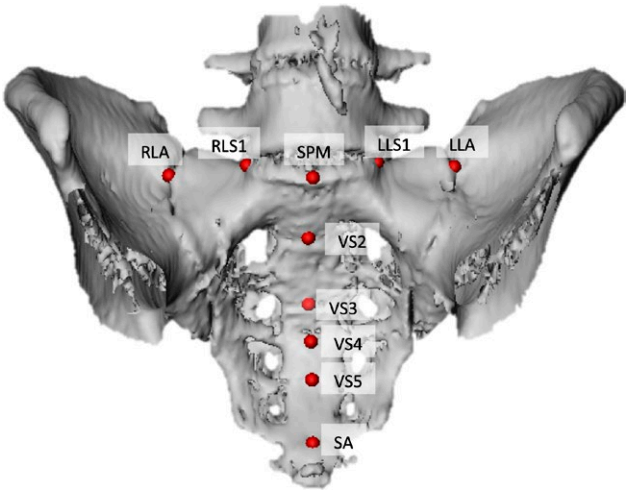

Figure 2. Anterior view of the sacrum with placed landmarks.

Table 2. Measurements taken between landmarks.

Measurements	Landmarks involved
Width of S1	Between RLS1 and LLS1
Length of the left ala	Between LLS1 and LLA
Length of the right ala	Between RLA and RLS1
Width of the sacrum	Between LLA and RLA
S1/ala breath ratio	Ratio between the width of S1 and the width of the sacrum and the ratio between the width of the alae and the width of the sacrum
Anterior height of the sacrum	Between SPM and SA

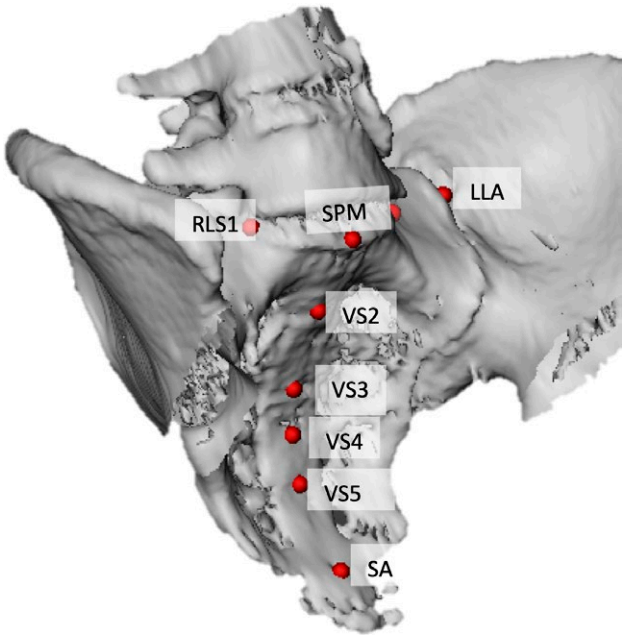

Figure 3. Antero-lateral view of the sacrum indicating the placed landmarks.

Table 3. Descriptive statistics of the dimensions of the ventral surface of the sacrum in a South African black sample.

Measurement (mm)	South African black females (n=26)	South African black males (n=25)	Differences (p-value)
Width of S1	**50.59** ± *7.34* (40.11–69.89)	**52.54** ± 5.10 (41.83–62.55)	0.28
Width of the ala	**107.33** ± 6.87 (92.19–120.35)	**108.14** ± 8.06 (80.15–118.48)	0.45
With of the right ala	**29.66** ± 3.76 (22.00–37.70)	**29.90** ± 3.48 (22.96–39.48)	0.82
Width of the left ala	**30.25** ± 4.06 (21.89–37.54)	**30.83** ± 4.76 (22.53–40.16)	0.65
Anterior height (height of the sacrum)	**99.88** ± 11.44 (79.13–126.32)	**108.49** ± 8.60 (89.81–123.20)	0.004*
S1/Ala ratio	**0.47** ± 0.06 (0.39–0.65)	**0.49** ± 0.04 (0.42–0.56)	0.08

* statistical significant difference ($p \leq 0.05$).

ent t-tests were performed to determine if significant sex-based differences exist between black South Africans for parametric data, while Kruskal-Wallis tests were used for non-parametric data. Basic descriptive statistics and sex-specific comparisons are summarised in Table 3.

Sacral height was significantly greater in black South African males compared to their female counterparts. A shorter and wide sacrum was expected as it contributes to the oval shaped pelvic outlet of females which promote vaginal delivery. Sacral width was slightly greater in black South African males, but with further investigation, it was noted that the female sacral width was generally greater, but exhibited more variation resulting in a smaller mean value. This can be verified, by looking at the landmark distribution as indicated on Figure 7.

The sex discriminative capacity of the sacrum measurements was evaluated using ROC curve analysis, as shown in Table 4. Anterior height demonstrated the highest sex discrimination accuracy, yielding a statistically significant Area Under the Curve (AUC = 0.73; 95% CI: 0.60 – 0.85; p = 0.001), with an optimal cut-off value of 103.41 mm (Sensitivity: 76 %, Specificity: 65.38 %), as shown in Figure 4. In contrast, the remaining sacral dimensions (Width of S1, Width of the ala, Width of the

Table 4. Receiver Operating Characteristic (ROC) curve analysis for estimating sex using sacral measurements.

Measurement (mm)	Area under the curve (AUC)	95% Confidence Interval (CI)	Differences (p-value)
Width of S1	0.61	0.47–0.75	0.28
Width of the ala	0.56	0.42–0.70	0.45
With of the right ala	0.51	0.37–0.66	0.82
Width of the left ala	0.54	0.40–0.69	0.65
Anterior height (height of the sacrum)	0.73	0.60–0.85	0.001*
S1/Ala ratio	0.64	0.50–0.78	0.08

* Statistical significant difference ($p \leq 0.05$).

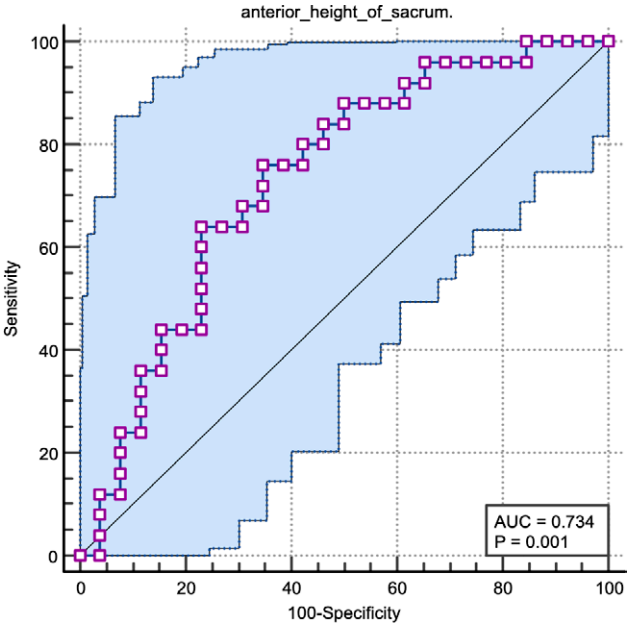

Figure 4. ROC curve analysis for sacral anterior height showing its diagnostic performance in discriminating between female and male individuals (AUC = 0.734, p = 0.001). The shaded area indicates 95% confidence interval.

right ala, Width of the left ala, and S1/Ala ratio) showed weak diagnostic performance, with AUC values ranging between 0.51 and 0.64, reflecting their lack of statistically significant differences between the sexes ($p > 0.05$).

To evaluate 3D shape variations of the ventral surface of the sacrum between sexes, a multivariate Principal Component Analyses (PCA) was performed. Principal Component 1 (PC1) accounted for 28.97% of the total variance, while PCA 2 accounted for 21.73%. The distribution and clustering of these scores are illustrated

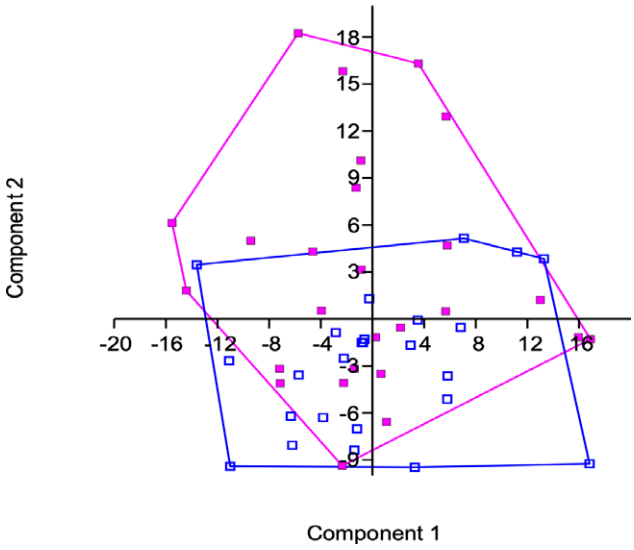

Figure 5. PCA graph indicating the variance in the shape of the ventral surface of the sacrum in black South African females (pink) and males (blue).

in Figure 5. In addition, a two-group permutation test confirmed a statistically significant difference in the ventral sacral shape between black South African males and females ($p = 0.005$).

Discriminant function analyses were performed on the 3D coordinates and linear distances to evaluate the capacity of ventral sacral shape to estimate sex. The discriminant models achieved a 94.12% classification accuracy when using 3D shape data (Fig. 6a) and a 72.55% accuracy when based on linear dimensions (Fig 6b).

DISCUSSION

In recent years, the sacrum has spiked the interest of scientist, with numerous studies demonstrating that its shape and dimensions are both sexually dimorphic and population-specific (Vollner et al., 2011; Iscan and Steyn, 2013; Rusk and Ousley, 2016; Lottering et al., 2022). Consequently, this study aimed to evaluate the efficacy of sex estimation within a black South African sample, using linear distances (measured between two 3D landmarks) and 3D shape coordinates on the ventral sacral surface.

Within this black South African sample, the sacral height varied significantly between the sexes ($p = 0.004$). Although the width of the of S1 was greater in males, it was not statistically significant. While the S1/Ala ratio (basewing index) is widely documented as sexual dimorphic in the literature (Rusk and Ousley, 2016), it did not vary significantly between males and females in this sample

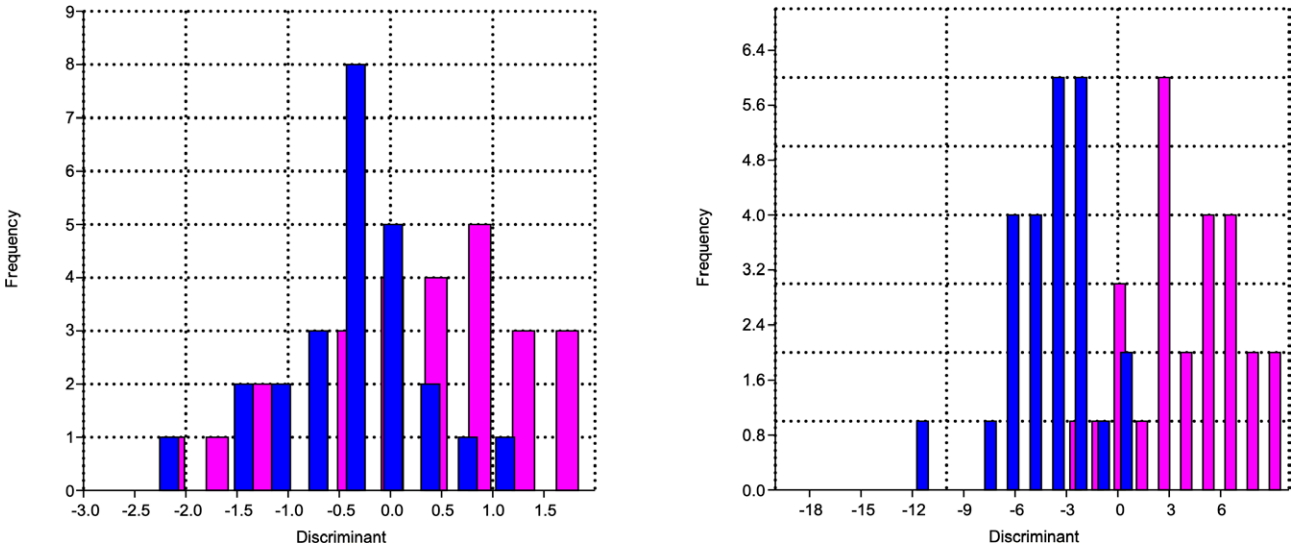

Figure 6. Bar graphs indicating the accuracy of sex determination using the ventral surface of the sacrum based on a) shape and b) linear dimensions

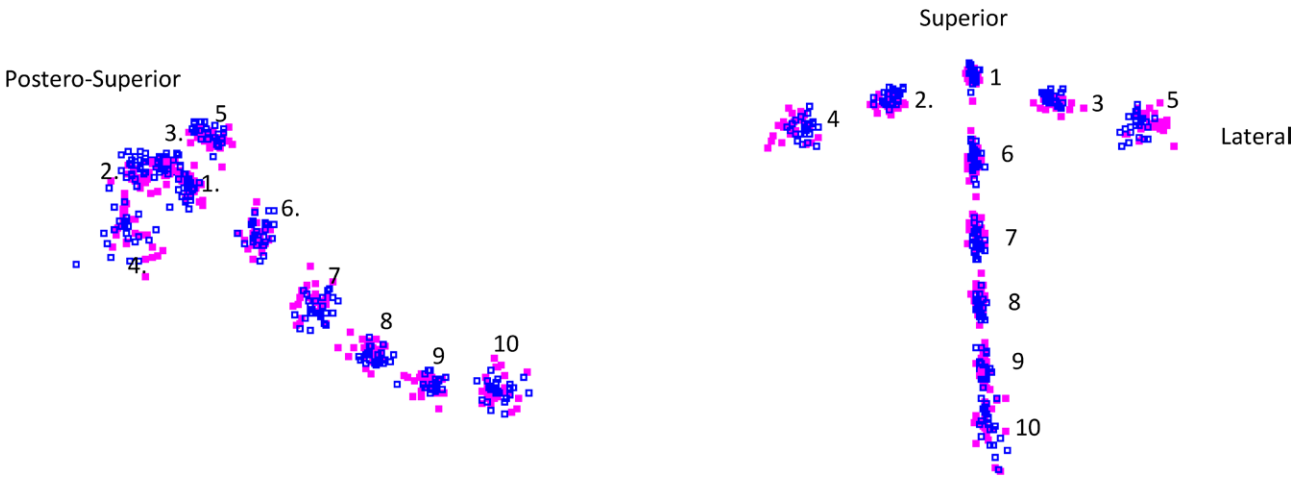

Figure 7. Procrustus coordinates and landmark position for South African black females (pink) and males (blue) from a) lateral view and b) anterior view indicating the dispersion of the landma.

Cross-population comparisons highlight distinct geomorphological variations. In the literature, Chinese individuals present with the shortest sacral length compared to all studied population groups (Zhan et al., 2018). While sacral length is sexually dimorphic in the black South African and Turkish samples, it displays no significant dimorphism in Japanese or American populations (Table 5). Conversely, sacral width is dimorphic in those global populations, but lacks dimorphism among black South Africans. The width of the sacral alae (sacral breath) does not vary between sexes in any of the populations summarised in Table 5, confirming its limited utility for sex estimation.

In this study, discriminant function analysis of the ventral sacral dimensions yielded a 72.55% accuracy for sex estimation, with males exhibiting a significantly greater sacral height than females. While this metric dimorphism is fully supported by Lottering and colleagues (2022), our classification accuracy is notably lower than the 88 - 94% achieved by Flander (1987). This discrepancy further underscores how linear skeletal dimensions can exhibit varying predictive reliability across different population groups. While ROC analysis

Table 5. Sacral dimension of various population groups based on literature.

Study:	Population:	Modality:	S1 width	Alae width	Sacral height	S1/ala ratio
This study	Black South Africans	CT	Female: 50.59±7.34 Male: 52.54 ± 5.1	Female: 108.14 ± 8.06 Male: 107.33 ± 6.87	Female: 99.88 ± 11.44* Male: 108.49 ± 8.6*	Female: 0.47 ± 0.06 Male: 0.49 ± 0.04
Zhan et al., 2018	Chinese	Multidetector CT			Female: 82.0 ± 6.2 Male: 88.3 ± 5.4	
Torimitsu et al., 2017	Japanese	Cadaver	Female: 52.25 ± 5.11* Male: 57.41 ± 5.77*	Female: 115.19 ± 5.60 Male: 116.98 ± 6.60	Female:102.58±10.52* Male:113.52±9.73*	
Rusk and Ousley, 2016	Black Americans	Sacral bone				Female:1/3:1/3;1/3 Males :1/4:1/2:1/4
Zech et al., 2012	Swiss	Sacral bone	Female:47.6±4,2 Male: 55.5±6.2	Female:115.1±6.1 Male: 114.8±5.1		
Plochocki, 2011	Americans	Sacral bones			Female:98.3±10.03 Male:100.1±8.00	
Pelin et al, 2005	Turkish males	MRI			Male: 109.5±12.1	
Flander, 1978	Black and white Americans	Sacral bones	BF:47.44±4.15* BM:54.5±4.28* WF:46.56±3.94* WM:52.78±5.21*	BF:111.36±7.23 BM:111.14±7.81 WF:117.62±7.08 WM:116.42±7.11	BF: 99.98±11.24 BM: 105.5±11.01 WF:109.64±12.56 WM:110.2±11.4	

* Statistically significant variations (p≤0.05), BF: Black female; BM: Black male; WF: White female; WM: White male.

substantiated the predictive value of these ventral linear dimensions with a significant Area Under the Curve (AUC: 0.76; CI 95%: 0.60 – 0.85; p = 0.004), the limitations of traditional linear metrics have increasingly driven researchers toward alternative methodologies.

Flander (1978) demonstrated that geometric morphometrics is a valuable method to determine sex from dry sacra after obtaining an 84% accuracy for white Americans and 91% accuracy for black Americans. In more recent years, Passalacqua and colleagues (2010) accurately determine sex from a black American sample, with 86% accuracy based on the shape of the sacrum defined by 3D landmarks. Notably, our results using3D coordinates of the ventral sacral surface yielded a 94,12 % accuracy in estimating sex of black South Africans, which demonstrates greater accuracy compared to the findings of Passalacqua and co-workers (2010).

Geometric morphometrics has been increasingly utilised for sex estimation in recent anthropological studies. Zech and colleagues (2012) achieved an 80 % sex prediction accuracy in a Swiss sample using CT scans. Conversely, the lowest sex classification accuracy, based on sacral shape was obtained by Franklin and co-workers (2014) who obtained a 70% classification accuracy using CT scans of an Australian sample.

Based on these studies conducted on across diverse population groups, sex can be estimated from the shape of the sacrum with an accuracy ranging between 70% - 94%. This overarching range demonstrates that shape-based analyses generally yield higher predictive rates than those based on traditional linear distances.

CONCLUSION

Knowledge of the anatomical variations of the sacrum between sex and population groups is integral in medical education, as it will influence the selection of operation techniques during surgical procedures and assist forensic scientists in human identification. This study demonstrates clear sexual dimorphism in the ventral surface of the sacrum, confirming its utility for accurately estimating sex in black South Africans.

The most effective method for sex estimation in this sample was based on 3D shape variation of the ventral surface of the sacrum, which has yielded an exceptional accuracy of 94,12 %. While linear distances measured across the ventral surface of the sacrum also successfully estimated sex with a 72,55 % accuracy rate, a finding supported by ROC analysis which confirmed a statistically significant Area Under the Curve (AUC = 0.73; 95% CI: 0.60 – 0.85; p = 0.004), shape analysis remains more accurate and reliable.

Consequently, it is recommended that forensic anthropologist and anatomical scientists prioritise 3D shape analysis of the ventral sacrum over traditional linear dimensions when estimating sex of skeletal remains within the black South African population.

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Beyond overload: A biofeedback-based assessment linking postural awareness and sensorimotor control to musculoskeletal health in orchestral musicians

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Abstract. Professional musicians often develop playing-related musculoskeletal disorders (PRMDs) from prolonged static postures, repetitive movements, and chronic biomechanical overload. However, the link between instrumental practice, postural awareness, and sensorimotor control lacks objective, multidimensional investigation. This preliminary study explored these relationships in 28 professional orchestral musicians divided into wind, bowed, and string instrument groups. Participants completed a structured questionnaire investigating musical practice habits, musculoskeletal symptoms, and postural awareness through the validated Postural Awareness Scale (PAS). Functional assessment included flexibility and core endurance tests, while sensorimotor control was quantitatively assessed, for the first time in this target, using the Libra biofeedback-based proprioceptive system. Musculoskeletal pain and discomfort were found to be highly prevalent across all groups, mainly involving the shoulder and spinal anatomical districts. Bowed instrument players showed significantly lower PAS scores than the other groups, suggesting reduced postural awareness associated with sustained asymmetric playing positions. Libra assessments revealed generally poor postural stability, particularly during eyes-closed conditions, indicating increased reliance on visual input for balance regulation and possible proprioceptive deficits. Functional tests also highlighted suboptimal core endurance and flexibility throughout the sample. Overall, findings support the hypothesis that PRMDs result from a complex interaction between cumulative biomechanical overload, reduced postural awareness, and altered sensorimotor control. In this context, specialized kinesiologists might play a pivotal role in prevention and management through tailored proprioceptive training, postural re-education, and adapted conditioning programs integrated into routine musical practice, thereby promoting long-term health, functional efficiency, and performance sustainability in this target population.

Keywords: postural awareness, proprioception, musculoskeletal disorders, biofeedback, adapted kinesiology, professional musicians.

INTRODUCTION

Musical performance requires a highly orchestrated integration of sensorimotor skills, postural regulation, sustained neuromuscular coordination, and complex cognitive processes (Belviso et al., 2026). The repetitive and specialized nature of instrumental practice exposes musicians to substantial physical demands, often comparable to those experienced by athletes in terms of training intensity, precision, and performance pressure (Quarrier, 1993; Ang, Panebianco & Odendaal, 2021; Creech & Hallam, 2003; Currey et al., 2020). However, unlike athletes, musicians often lack structured preventive training, increasing their susceptibility to playing-related musculoskeletal disorders (PRMDs) (Foxman & Burgel, 2006; Chan & Ackermann, 2014; Baadjou et al., 2015). PRMDs, defined as pain, weakness, numbness, or functional impairment affecting performance, represent one of the most prevalent occupational health issues among musicians (Gasenzer et al., 2017; Blanco-Piñeiro, Díaz-Pereira & Martínez, 2017; Cerda-Díaz et al., 2026; Shinde & Borkar, 2021), with consistently high prevalence rates reported across different instrumental groups (Kok et al., 2016; Rotter et al., 2020; Cerda-Díaz et al., 2026). The cervical spine, shoulder girdle, and upper limbs seem to be particularly involved, reflecting the specific biomechanical constraints imposed by instrumental performance (Silva, Lã & Afreixo, 2015; Gómez-Rodríguez et al., 2020; Kochem & Silva, 2018; Steinmetz, Seidel & Muche, 2010). The etiology of PRMDs is complex and multifactorial. Traditional models have emphasized mechanical overload, repetitive strain, and sustained static postures as primary risk factors (Baadjou et al., 2016; Betzl, Kraneburg & Megerle, 2020).

From a biomechanical perspective, professional instrumental practice is characterized by a unique combination of prolonged static loading, highly repetitive fine motor activity, and asymmetric postural configurations, which together generate a distinctive pattern of cumulative mechanical overload. Musicians are required to maintain submaximal but sustained muscle activation over extended periods, often in constrained joint positions that limit physiological variability and recovery (Kok et al., 2017; Rensing, Schemmann & Zalpour, 2018). These conditions promote the development of low-grade chronic fatigue, reduced local circulation, and progressive accumulation of microtrauma within musculoskeletal tissues (Fry, 1986; Sirufo et al., 2022). Instrument-specific demands further exacerbate these biomechanical stresses. For instance, bowed string players typically adopt persistent cervical rotation, lateral flexion, and elevation of the shoulder, associated with prolonged scapular stabilization and asymmetric loading of paravertebral and shoulder girdle muscles (Steinmetz, Seidel & Muche, 2010; Barczyk-Pawelec et al., 2012; Saffert, Melzner & Dendorfer, 2022; Ancillao et al., 2017; Rensing, Schemmann & Zalpour, 2018). Pianists and harpists are exposed to repetitive high-frequency finger movements combined with prolonged sitting and static trunk posture, resulting in continuous activation of both distal and proximal kinetic chains (Ciurana Moñino et al., 2017; Furuya et al., 2011; Chadefaux et al., 2024). Wind instrument players require sustained respiratory effort and increased activation of core musculature, contributing to thoraco-lumbar stabilization demands and altered pressure dynamics (Ackermann & Driscoll, 2010; Chan, Driscoll & Ackermann, 2013; López-Pineda et al., 2023). These conditions lead to non-ergonomic joint alignments and altered load distribution, promoting biomechanical imbalances and maladaptive structural changes over time (Blanco-Piñeiro, Díaz-Pereira & Martínez, 2017; Fernández Paz, Lantarón Caeiro & Soto González, 2020; Rousseau et al., 2023; Barczyk-Pawelec et al., 2012). The coexistence of repetition, force, and static posture creates a cumulative overload scenario in which tissue recovery is insufficient relative to exposure (Rotter et al., 2020; Betzl, Kraneburg & Megerle, 2020). Furthermore, the high level of motor precision required in musical execution tends to reduce movement variability that is a factor associated with increased risk of overuse (Turner et al., 2023). Reduced variability, combined with performance demands and error minimization, may therefore contribute to persistent stereotyped motor patterns and localized overload. At the neuromuscular level, prolonged exposure to these constraints may induce adaptive and maladaptive changes in motor control strategies. Altered muscle recruitment patterns, increased co-contraction, and inefficient coordination between synergistic muscle groups have been observed in musicians with PRMDs (Overton, Du Plessis & Sole, 2018; Silva et al., 2018). These changes likely reflect both peripheral fatigue and central adaptations, suggesting a reorganization of sensorimotor control in response to repetitive strain (Mizrahi, 2020). In this context, proprioception and sensorimotor integration play a crucial role in optimizing postural control and movement efficiency. Accurate proprioceptive input contributes to body schema representation, regulation of postural tone, and fine motor control (Steinmetz, Seidel & Muche, 2010; Smitt & Bird, 2013). Conversely, altered proprioceptive processing may lead to deficient postural strategies, increased muscular stiffness, and suboptimal load distribution, ultimately amplifying biomechanical stress (Carretti, Manetti & Marini, 2025a; Steinmetz, Seidel & Muche, 2010). Emerging evidence has also indicated that musicians with PRMDs exhibit impairments in postural

control systems (Rodríguez-Gude, Taboada-Iglesias & Pino-Juste, 2023), further supporting the relevance of sensorimotor control in this target of individuals (Steinmetz, Seidel & Muche, 2010; Garnett, Pijl & Visentin, 2022).

Despite the growing body of literature addressing occupational health risks in musicians, the relationship between biomechanical overload and sensorimotor control alterations remains insufficiently clarified. Most available studies have predominantly focused on epidemiological prevalence, self-reported symptoms, or isolated risk factors, often relying on questionnaire-based approaches without integrating objective functional assessments (Baadjou et al., 2016; Stanhope et al., 2019; Rodríguez-Gude, Taboada-Iglesias & Pino-Juste, 2023). Whereas these contributions have been essential in defining and highlighting the magnitude of the problem, they provide limited insight into the mechanisms linking repetitive biomechanical exposure to altered motor control strategies (Cahalan et al., 2026; Rousseau et al., 2023). Moreover, although postural and ergonomic analyses have been widely investigated, they have been rarely integrated with proprioceptive and sensorimotor dimensions, which are essential for understanding movement regulation in highly skilled performers (Rousseau et al., 2023; Garnett, Pijl & Visentin, 2022). Similarly, neuromuscular studies have documented altered muscle activation patterns in musicians with PRMDs, yet these findings resulted poorly contextualized within a comprehensive framework that includes postural control, sensory integration, and task-specific motor behavior (Overton, Du Plessis & Sole, 2018; Silva et al., 2018). Another important limitation concerns the scarcity of multimodal and quantitative assessment approaches capable of capturing the complexity of instrumental performance. Existing methodologies often rely either on laboratory-based biomechanical analyses with limited ecological validity or on clinical observational tools that may lack sensitivity to detect subtle or early-stage dysfunctions (Ancillao et al., 2017; Schemmann, Rensing & Zalpour, 2018). Consequently, the transition from adaptive motor strategies to maladaptive patterns leading to injury remains poorly understood. In addition, the role of practice-related variables, such as intensity, duration, and cumulative exposure, has been widely acknowledged but insufficiently integrated with objective measures of motor control and postural regulation, hence limiting potential meaningful associations between behavioral load and functional impairment (Kok et al., 2016; Stanhope, Weinstein & Pisaniello, 2020). This gap is particularly relevant in orchestral musicians, who are exposed to prolonged and often non-modifiable performance demands. Therefore, a comprehensive approach that simultaneously considers biomechanical load, sensorimotor control, and related symptomatology is still lacking.

The present preliminary study aims at addressing this gap by adopting a multidimensional assessment framework that combined validated qualitative instruments, standardized motor evaluations, and an innovative biofeedback-based tool to objectively investigate sensorimotor control in relation to musculoskeletal symptoms and practice habits in this target population. This approach might hopefully foster research interest in this still under investigated field through a more mechanistic understanding of PRMDs and contemporarily provide kinesiological preventive/re-educative hints to be stably integrated into instrumental training sessions. Such desirable integration might allow a tailored holistic management of this highly specialized population, thereby contributing to effectively prevent and counteract occupational health issues while safely improving quality of life, artistic performance and long-term career.

MATERIALS AND METHODS

Participants

The investigated sample comprised 28 musicians, 14 women and 14 men, aged between 21 and 64 years. All participants were professional musicians regularly enrolled in formal musical training programs and/or belonging to orchestras, with an academic background from a conservatoire. Specifically, the study included orchestral musicians with professional experience in instrumental practice, no history of neurological or musculoskeletal disease, and who provided informed consent to adhere to the study. The study was conducted in accordance with the principles of the Declaration of Helsinki of 1975, revised in 2013 (World Medical Association, 2013). Individuals reporting acute pain and recent trauma or surgery at the time of assessment were excluded since such issues might jeopardize balance and safety during sensorimotor tasks. To identify how instrument-specific postures affect sensorimotor control, participants were grouped by instrument type: wind, bowed, and other string instruments. Notably, the string instruments category comprised both harp and harpsichord players since both instruments share a common physical sound-production mechanism based on string plucking – either directly or via a keyboard mechanism – rather than bowing or wind generation. Musicians were recruited through direct contact with various orchestras/music institutions and voluntary participation in the study was promoted via their official communication channels. During this preliminary phase, objectives, operational

procedures, organizational and logistical aspects were clearly presented and detailed by an adapted physical activity kinesiologist and approved by the internal review board of each involved music institution. This ensured a clear understanding of the procedures involved prior to the study, promoting informed and responsible participation. In the act of adhering to the study, participants deliberately and consciously gave their consent not only to participate in the planned procedures, but also to data collection, storage, and utilization for scientific publications. In agreement with this form, data were treated, processed, and stored in a completely anonymous way for the purposes of this study. In particular, the obtained information was entered into an electronic datasheet identifying each participant by a unique alphanumeric ID, not mentioning, or making any sensitive data accessible. Given the relatively limited sample size, particularly in the string instrument subgroup, the present investigation should be considered a preliminary exploratory study aimed at generating initial evidence and methodological hints for future larger-scale research.

Qualitative and quantitative evaluations

All study participants filled out a structured questionnaire purposely designed using the Google Forms platform and sent by e-mail in the form of a direct access link to the form. In detail, the first section of the self-administered survey was aimed at collecting sociodemographic and anthropometric data, including age, gender, dominant hand, weight, height and relevant characteristics of musical practice (e.g., played instrument, years of experience, weekly time spent practicing). Regarding the anthropometric aspect, based on the weight and height data provided by each participant, the body mass index was calculated using the formula weight (kg)/height (m^2). The second section examined habits related to instrumental practice, including information regarding the duration of training sessions, the inclusion of warm-up exercises and/or breaks during practice, and any postural training received. Specific purpose-designed questions investigated the self-perception of posture during musical training/performance, as well as the subjective postural awareness. Additional questions were then aimed to identify the presence of pain or physical discomfort associated with musical practice, its frequency and location, or any experienced postural/musculoskeletal issues. Information regarding the level of habitual physical activity and sedentary lifestyle was also collected. Finally, the questionnaire included a section dedicated to assessing postural awareness using the Italian version of the Postural Awareness Scale (PAS), a validated tool designed to investigate the level of subjective perception of postural control in daily activities. The scale consists of 12 items designed to explore both spontaneous and conscious postural awareness. The total score ranges from 12 to 84, with higher values indicating greater sensorimotor awareness (Topino, Gori & Cramer, 2020).

After completing the questionnaire, participants underwent a battery of evidence-based motor tests aimed at assessing flexibility, muscular endurance and core stability/strength. All the tests, performed strictly following the official guidelines available in literature, were accurately detailed and then supervised by a specialized kinesiologist. In particular, the V Sit-and-Reach test was administered to assess flexibility of the posterior kinetic chain, peculiarly the hamstring musculature and lower back extensibility. Such evaluative tool is considered a valid field-based alternative to the traditional version of the test, minimizing spinal compression and improving standardization (Hui & Yuen, 2000; Miñarro et al., 2007). Detailing procedures, each participant was asked to assume a seated position on the floor with lower limbs extended and abducted at approximately 30–45°, forming a "V" shape. The heels placed against a baseline marked on the floor, with the zero-point aligned with the heels. The measuring line extended forward along the midline between lower limbs. The subject was requested to place one hand over the other, with arms fully extended, and slowly reach forward along the measuring line, maintaining knee extension, avoiding ballistic movements, and holding the final position for at least 3 seconds. The distance reached beyond the baseline was measured in centimeters. Three attempts were made retaining the best reached value and then comparing it with the reference scores available in literature (Hui & Yuen, 2000; Miñarro et al., 2007). The Sit-Up test was performed as well to evaluate dynamic muscular endurance of the abdominal musculature, particularly the rectus abdominis and hip flexors (Kukić et al., 2022; Bianco et al., 2015). The subject was asked to lie in supine decubitus position on a fitness mat placed on the floor, with bent lower limbs and feet kept parallel and hip-width apart, firmly on the ground. Placing arms outstretched and parallel, hands resting on thighs with palms facing the floor, participant was requested to keep shoulders and head on the mat. From this position, shoulders and upper body had to be lifted just enough to allow the fingertips of both hands rising above knee height and then return to the starting position. The aim of the test was to perform as many correctly executed repetitions as possible in one minute. The achieved reps were then compared with the specific cut-off available in

literature (Kukić et al., 2022; Bianco et al., 2015). Subsequently, plank tests were also administered to assess isometric endurance of the core musculature, including rectus and transverse abdominis, obliques, and stabilizing muscles of the lumbar-pelvic region (Tong, Wu & Nie, 2014; Rodríguez-Perea et al., 2025). Specifically, regarding the front plank test, each participant was asked to assume a prone decubitus position, supporting body weight on the forearms and toes. Elbows aligned vertically under the shoulders, forearms parallel, and the body maintained in a straight line from head to heels. The subject was instructed to hold this position as long as possible without compromising alignment (e.g., pelvic drop, excessive lumbar lordosis, or scapular instability). Time was recorded in seconds starting from the moment the correct position was assumed until failure, defined as the inability to maintain the required proper posture (Rodríguez-Perea et al., 2025). Similarly, in the side plank test subject was asked to lie on the floor in lateral decubitus position, supporting the body on one forearm and the lateral face of the foot. The elbow was positioned directly under the shoulder, and the body had to be kept aligned in a straight line. The contralateral arm was placed along the body or on the hip. Both left and right sides were tested by recording time independently for each side, applying the same criteria previously detailed. Reference cut-off values are available in literature for both these tests (Tong, Wu & Nie, 2014).

Finally, the efficiency of sensorimotor control was tested using the Libra sensorized proprioceptive board (Carretti, Manetti & Marini, 2025b), an innovative biofeedback based device designed to provide an accurate and reliable quantitative assessment (Tchórzewski, Jaworski & Bujas, 2010). Specifically, it consists of a sensorized tilting board connectable, via USB, to any computer and specific software interface. The tool is equipped with interchangeable rolling wedges that allow variable oscillation degrees while, through the digital interface, different path patterns can be set and then displayed on the computer screen as a roadway side bounded by two parallel lines. During the swing, whenever such boundaries are approached or overcome, a multimodal feedback is played to signal the departure from the equilibrium position (Tchórzewski, Jaworski & Bujas, 2010). The digital interface also offers a validated preset test called Spielman-De Gunsch (SDG) Test. Such validated trial allows to assess the global postural stability in orthostatic bipedal stance, related to different conditions of visual anchorage: no gaze constraints, straight ahead gaze, and closed eyes (Adamo, Pociask & Goldberg, 2013). During the SDG Test the subject was asked to stand up in orthostatic position on Libra trying to control oscillations by keeping it as parallel to the floor as possible. Specifically, the test includes three consecutive 30-second tasks performed following a linear pathway pattern, presetting a 9/10 difficulty level and 10 cm tilting wedges. At the end of the whole execution, the stability index obtained in each trial can be compared to the 0–100 cut-off values available in the digital database, with lower scores corresponding to higher postural stability (Carretti, Manetti & Marini, 2025b). Clinically, the validated stability index reflects the ability to control postural sway and maintain balance under micro/macro perturbations and different visual anchorage conditions. Therefore, this parameter may represent a crucial outcome for investigating the multimodal complex sensorimotor control of professional musicians. Furthermore, sensorimotor control of the upper body district was investigated using the Libra board in seated position, a specific setting foreseen by the manufacturer and already applied and described in our previous studies (Carretti et al., 2024a, 2024b). In line with the aim of such assessment, Libra was placed on a stable and anti-slip box, and the subject was instructed to sit over it, keeping arms crossed over chest, with a 90◦ trunk-thigh angle and both legs orthogonal to the ground with feet on a skimmy proprioceptive cushion on the floor. Starting from this position, two tasks were performed, one allowing the board to tilt on frontal plane while the other on sagittal plane. Such variability, simply achieved by changing the Libra positioning from straight-oriented to transverse-oriented, was purposely applied to investigate trunk lateral and antero-posterior stability, respectively (Carretti et al., 2024b). Both tests lasted 1 min and a linear pathway pattern, a maximum difficulty level, and 10 cm tilting wedges were set. Such seated trials were carried out either without the habitually played instrument or in a playing simulated setting assuming the required position. To ensure safety and protect valuable musical instruments, actual instrument use was avoided during these tests. As already detailed for SDG Test, the aim was to keep Libra in balance, parallel to the floor, following the multimodal feedback provided by the digital interface. The achieved performance index was then interpreted using the validated 0–100 cut-off.

Statistical analysis

GraphPad Prism 5 software was used to conduct statistical analysis. One-way analysis of variance (ANOVA) and posthoc Tukey's multiple comparison test were used to compare study groups after Kolmogorov–Smirnov test confirmed the normality of data, which were stated as mean ± standard deviation (SD). Chi-square test was also

used for categorical data, which were stated as number/percentage of subjects participating in the study. A p-value of less than 0.05 was deemed statistically significant.

RESULTS

The demographic and anthropometric characteristics of the study participants are detailed in Table 1. Considering the musical instrument played, half of the study sample played bowed instruments, followed by wind instruments and, to a lesser extent, other string instruments such as harp and harpsichord. No statistically significant difference between the three groups (wind instruments, bowed instruments, and string instruments) was observed for the main variables investigated. Specifically, the age-distribution was balanced and there was no difference in the BMI category. Notably, most participants were normal weight, whilst smaller proportion were overweight or underweight. Table 1 summarizes the results related to the current practice of regular physical activity/sport, assessed by question-

Table 1. Demographic/anthropometric data and summary data from questionnaire investigating the current practice of regular physical activity/sport for each of the three study groups.

Variables	Wind instruments (n = 11)	Bowed instruments (n = 14)	String instruments (n = 3)
Age (years), mean ± SD (range)	29.73 ± 8.83 (22–54)	29.57 ± 12.46 (21–64)	29.67 ± 3.51 (26–33)
Sex, n (%)			
Male	7 (63.64)	6 (42.86)	1 (33.33)
Female	4 (36.36)	8 (57.14)	2 (66.67)
BMI category, n (%)			
<18.5 (underweight)	1 (9.09)	2 (14.29)	0 (0.00)
18.5–24.9 (normal weight)	7 (63.64)	6 (42.86)	3 (100.00)
25–29.9 (overweight)	3 (27.27)	6 (42.86)	0 (0.00)
≥ 30 (obese)	0 (0.00)	0 (0.00)	0 (0.00)
Current physical activity/sport practice, n (%)			
Yes	8 (72.73)	7 (50.00)	1 (33.33)
No	3 (27.27)	7 (50.00)	2 (66.67)
Type of physical activity/sport, n			
Walking	4	4	0
Running	1	2	0
Gym	3	2	0
Yoga/Pilates	3	2	1
Swimming	1	1	0
Team sports	0	2	0
Physical activity/sport frequency per week (days), n			
<1	0	4	0
1–2	4	3	0
3–4	1	0	1
7	3	0	0
Average duration of physical activity/sport session (minutes), n			
<30	1	0	1
30–60	7	5	0
>60	0	2	0
Average daily hours spent sitting, n (%)			
1–3	1	1	0
4–6	5	8	2
7–10	5	3	1
Unsure	0	2	0

BMI, body mass index; SD, standard deviation.

naire, with no significant differences observed between three study groups. In particular, most participants reported engaging in regular physical activity or sports, such as walking, running, gym workouts, yoga/Pilates, or swimming. Finally, the range 4–6 hours mainly represented the amount of daily time spent sitting.

Results of postural habits, awareness, and pain associated with musical practice as well as presence of musculoskeletal complaints are shown in Table 2. Overall, the sample comprised musicians with at least 10 years of experience who practice daily. Most musicians reported musculoskeletal pain presence mainly localized to the shoulder or spine, due to the time spent playing and the posture adopted while performing. In particular, the three groups did not differ significantly in their responses. However, it is notable that significant differences in self-reported postural awareness score, assessed by PAS, were found in bowed instrument players compared to wind and string musicians ($p < 0.05$ respectively). In detail, bowed instrument players showed lower postural awareness scores (Table 2).

Furthermore, most participants reported prolonged daily practice sessions with limited interruption hence recalling the presence of cumulative biomechanical overload associated with instrumental performance (data not shown).

Table 3 shows the data of postural stability, assessed by Libra, and the results of functional assessment in the study groups. In particular, the global postural stability, tested using Libra SDG in orthostatic bipedal stance, related to different conditions of visual anchorage (i.e., no gaze constraints, straight ahead gaze, and closed eyes) highlighted poor levels of sensorimotor control in all study groups. Specifically, postural stability tended to worsen during eyes-closed conditions, showing a greater dependence on visual input for balance regulation. Similarly, the trunk stability index in seated position on both frontal and sagittal planes, with and without the musical instrument, did not reveal any statistically significant differences among the three groups of musicians. Moreover, the posterior muscle chain flexibility, measured by the sit-and-reach test, and core stability assessed by plank tests, showed no statistically significant differences among the three study groups. According to the available validated cut-off values, these collective data indicated a poor level of physical fitness. Although overall core endurance values were generally suboptimal across the sample, wind instrument players tended to demonstrate slightly better core strength and endurance performances compared with the other groups.

DISCUSSION

To our knowledge, the present study is the first to provide a multidimensional insight into the complex interaction between biomechanical load, postural habits, and sensorimotor control in orchestral musicians, supporting the hypothesis that these factors are strictly interconnected and collectively contribute to the development of PRMDs. The high prevalence of self-reported pain and discomfort observed in all the instrumental groups investigated is consistent with the extensive literature documenting musculoskeletal complaints as one of the most common occupational health issues in musicians (Kok et al., 2016; Rotter et al., 2020; Gómez-Rodríguez et al., 2020; Cerda-Díaz et al., 2026). In particular, the predominance of shoulder, cervical, and spinal involvement aligns with previous findings highlighting the upper body as the most exposed district due to sustained asymmetric postures and repetitive motor patterns (Silva, Lã & Afreixo, 2015; Kochem & Silva, 2018; Gómez-Rodríguez et al., 2020). From a behavioral perspective, the data collected through the questionnaire revealed a substantial exposure to risk factors traditionally associated with PRMDs, including prolonged daily practice, limited variability in posture, and high weekly playing frequency. Notably, most musicians reported practicing 4-6 hours per day with minimal interruption, reinforcing the link between cumulative overload and insufficient recovery time described in earlier studies (Baadjou et al., 2016; Betzl, Kraneburg & Megerle, 2020). The observed association between practice duration and impaired postural control highlighted the role of training load. Prolonged practice without adequate recovery leads to neuromuscular fatigue, which impairs proprioceptive acuity and increase postural sway. This finding is aligned with research indicating that workload management represents a critical factor in the prevention of PRMDs (Baadjou et al., 2016; Amaral Corrêa et al., 2018). Additionally, the frequent adoption of non-neutral trunk and head alignments, such as forward inclination, lateral flexion, and rotation, confirmed the persistence of instrument-specific postural constraints already documented in the literature (Barczyk-Pawelec et al., 2012; Fernández Paz, Lantarón Caeiro & Soto González, 2020; Rousseau et al., 2023). These maladaptive postures, when maintained over time, may alter load distribution and contribute to microtrauma accumulation, ultimately leading to chronic dysfunctions (Fry, 1986; Sirufo et al., 2022). In fact, it has been demonstrated that instrument-specific biomechanical constraints play a crucial role in shaping these adaptations, significantly compromising proprioception efficiency and postural control (Barczyk-

Table 2. Summary data from questionnaire focusing on the postural habits, awareness, and pain associated with musical practice as well as presence of musculoskeletal complaints.

Variables	Wind instruments (n = 11)	Bowed instruments (n = 14)	String instruments (n = 3)
Handedness, n (%)			
Right-handed	9 (81.82)	13 (92.86)	3 (100.00)
Left-handed	1 (9.09)	1 (7.14)	0 (0.00)
Mixed-handed	1 (9.09)	0 (0.00)	0 (0.00)
Years of musical experience, n (%)			
1–9	1 (9.09)	1 (7.14)	0 (0.00)
10–20	6 (54.55)	10 (71.43)	2 (66.67)
> 20	4 (36.36)	3 (21.43)	1 (33.33)
Weekly frequency of playing on a daily, n (%)			
1–2	0 (0.00)	0 (0.00)	0 (0.00)
3–4	0 (0.00)	2 (14.29)	0 (0.00)
5–6	4 (36.36)	4 (28.57)	0 (0.00)
7	7 (63.64)	8 (57.14)	3 (100.00)
Average hours of musical playing, n (%)			
< 1	0 (0.00)	0 (0.00)	0 (0.00)
1–2	3 (27.27)	2 (14.29)	0 (0.00)
3–4	6 (54.55)	7 (50.00)	2 (66.67)
5–6	2 (18.18)	5 (35.71)	0 (0.00)
> 6	0 (0.00)	0 (0.00)	1 (33.33)
Posture adopted while playing, n (%)			
Sitting	7 (63.64)	5 (35.71)	3 (100.00)
Static standing	4 (36.36)	3 (21.43)	0 (0.00)
Dynamic standing	0 (0.00)	6 (42.86)	0 (0.00)
Trunk alignment while sitting playing, n			
Straight	1	2	2
Slightly leaning forwards	5	2	1
Slightly leaning backwards	1	1	0
No attention to posture	0	0	0
Trunk alignment while standing playing, n			
Straight and aligned	2	1	0
Flexion	0	3	0
Lateral flexion	2	1	0
Unbalanced to one side	0	4	0
No attention to posture	0	0	0
Head alignment (orientation) while playing, n (%)			
Neutral position	4 (36.36)	2 (14.29)	0 (0.00)
Flexion	5 (45.45)	1 (7.14)	2 (66.67)
Lateral flexion	2 (18.18)	6 (42.86)	1 (33.33)
Rotation	0 (0.00)	4 (28.57)	0 (0.00)
No attention to posture	0 (0.00)	1 (7.14)	0 (0.00)
Pain/discomfort presence, n (%)			
Yes	8 (72.73)	12 (85.71)	2 (66.67)
No	3 (27.27)	2 (14.29)	1 (33.33)
Body localization, n			
Neck	3	4	0
Shoulder	8	7	1
Spine	2	7	0
Arm	0	3	0
Wrist	2	1	1
Hand	1	2	0

(Continued)

Table 2. (Continued).

Variables	Wind instruments (n = 11)	Bowed instruments (n = 14)	String instruments (n = 3)
Pain/discomfort onset frequency, n			
Always	1	3	0
Often	6	5	1
Rarely	1	4	1
Never	0	0	0
Osteoarticular disorder presence, n (%)			
Yes	4 (36.36)	5 (35.71)	0 (0.00)
No	7 (63.64)	9 (64.29)	3 (100.00)
Symptom presence in the last month, n (%)			
Yes	2 (50.00)	3 (60.00)	0 (0.00)
No	2 (50.00)	2 (40.00)	0 (0.00)
Symptom presence in the past week, n (%)			
Yes	2 (50.00)	3 (60.00)	0 (0.00)
No	2 (50.00)	2 (40.00)	0 (0.00)
Functional impact on instrumental performance, n (%)			
Yes	3 (75.00)	5 (100.00)	0 (0.00)
No	1 (25.00)	0 (0.00)	0 (0.00)
Symptom attribution to the instrument playing, n (%)			
Yes	3 (75.00)	2 (40.00)	0 (0.00)
No	0 (0.00)	1 (20.00)	0 (0.00)
Unsure	1 (25.00)	2 (40.00)	0 (0.00)
Postural Awareness Scale (total score), mean (SD)	52.81 (9.90)	41.85 (11.12)*	60 (8.88)

SD, standard deviation; *$p < 0.05$ compared to wind and string instruments.

Table 3. Mean scores of anatomo-functional and sensorimotor control assessments for each of the three study groups.

Variables	Wind instruments Mean ± SD	Bowed instruments Mean ± SD	String instruments Mean ± SD
Libra SDG test, index			
No gaze constraint	17.14 ± 1.83	16.07 ± 1.79	16.82 ± 1.96
Straight ahead gaze	16.20 ± 2.17	15.51 ± 2.96	15.94 ± 0.42
Closed eyes	17.96 ± 1.91	17.76 ± 1.59	17.18 ± 0.72
Libra seated tests, index			
Frontal plane trunk stability	0.78 ± 1.05	0.48 ± 0.23	0.48 ± 0.23
Frontal plane trunk stability with musical instrument	0.96 ± 1.85	0.42 ± 0.21	0.35 ± 0.05
Sagittal plane trunk stability	1.77 ± 4.70	0.40 ± 0.58	0.47 ± 0.46
Sagittal plane trunk stability with musical instrument	0.55 ± 0.89	0.40 ± 0.58	0.35 ± 0.18
V Sit-and-reach test, centimeters	41.54 ± 14.27	34.93 ± 17.12	31.43 ± 11.57
Sit-up, n repetition/min	23.00 ± 7.80	20.71 ± 8.64	16.66 ± 2.08
Plank, seconds			
Front	100.72 ± 37.55	79.28 ± 42.76	92 ± 28.79
Right	48.09 ± 22.89	43.71 ± 24.95	33.33 ± 5.5
Left	54.36 ± 20.03	41 ± 25.63	44.66 ± 16.92

SDG, Spielman-De Gunsch; SD, standard deviation.

Pawelec et al., 2012; Blanco-Piñeiro, Díaz-Pereira & Martínez, 2017; Ancillao et al., 2017; Rensing, Schemmann & Zalpour, 2018). Specifically concerning postural awareness, measured by PAS, the relatively heterogeneous scores observed among the instrumental groups investigated suggested that subjective perception of posture may vary depending on instrument-specific demands. In particular, bowed instrument players showed lower postural awareness scores, suggesting a possible association between sustained asymmetric playing positions and reduced subjective perception of posture and body alignment. This aspect is particularly relevant considering that reduced body awareness has been associated with inefficient motor strategies and increased injury risk (Smitt & Bird, 2013; Garnett, Pijl & Visentin, 2022). The gap between high biomechanical stress and moderate postural awareness may impair self-regulation, perpetuating harmful motor habits in this target of individuals. The functional motor assessments by validated tasks further supported such interpretation. Although flexibility and core endurance values did not show statistically significant differences between groups, a general trend toward suboptimal performance, particularly in core stability tests, can be observed. Despite this overall deficit, wind instrument players showed better values in core strength and endurance thus remarking the link between instrument-specific posture and somatosensory adjustments. Given that core parameters are essential for maintaining postural control and distributing mechanical loads efficiently, a conscious recruitment of this anatomo-functional district becomes crucial during instrumental performance (Ackermann & Driscoll, 2010; Chan, Driscoll & Ackermann, 2013). Insufficient conditioning of stabilizing musculature may thus exacerbate compensatory strategies and increase local stress on peripheral segments, as also suggested by studies demonstrating the beneficial effects of targeted exercise programs in reducing pain and improving function in musicians (Lundborg & Grooten, 2018; Mirshahi et al., 2023).

The main innovative contribution of the present study lies in the objective assessment of sensorimotor control through the validated biofeedback-based Libra system. The relatively homogeneous SDG test scores across groups suggest a generally preserved global postural stability under visual conditions. Conversely, during eyes-closed tasks, altered postural control patterns have been evidenced, hence suggesting that musicians might exhibit deficits in proprioceptive integration, relying more heavily on visual input to maintain stability. Similar findings have been reported in individuals with chronic musculoskeletal pain, where sensory reweighting mechanisms become altered, leading to reduced adaptability (Rodríguez-Gude, Taboada-Iglesias & Pino-Juste, 2023). From a neurophysiological standpoint, these alterations may reflect changes in central processing of sensory information. Indeed, prolonged exposure to repetitive and constrained motor tasks can lead to plastic adaptations within the central nervous system, potentially reinforcing suboptimal movement patterns (Overton, Du Plessis & Sole, 2018; Belviso et al., 2026). Contemporarily, slight differences emerged when analyzing trunk stability during dynamic seated tasks on Libra board, particularly when comparing conditions with and without simulated instrument playing. The observed variability in the performance index, with and without playing simulation, may reflect the task-related adaptations to complex postural demands imposed by instrument-specific positions. These findings support the hypothesis that sensorimotor control is task-dependent and may lead to maladaptive adjustments under ecologically relevant conditions, where biomechanical and cognitive demands are combined for extended periods (Steinmetz, Seidel & Muche, 2010; Mizrahi, 2020). In this context, the increased stability index during not playing conditions can be interpreted as an indicator of reduced adaptability of the sensorimotor system when exposed to habitual featured constraints (Overton, Du Plessis & Sole, 2018; Silva et al., 2018). Furthermore, the relationship between pain and impaired postural control observed in the investigated sample highlighted a bidirectional interaction. Pain can modify motor output through protective mechanisms, such as increased stiffness and reduced movement variability, which in turn may increase mechanical stress on tissues (Betzl, Kraneburg & Megerle, 2020). This dangerous vicious cycle might contribute to the persistence and chronicity of symptoms. Finally, a relevant aspect concerns the relationship between physical activity and musculoskeletal health. Although a relatively high proportion of participants reported engaging in regular physical activity, this did not appear sufficient to prevent the occurrence of pain and discomfort. Consistent with previous studies, this finding suggests that generic physical activity alone might not be adequate to counteract the specific biomechanical demands of musical performance (Baadjou et al., 2015; Roos et al., 2024). Instead, tailored kinesiologist-led strategies focusing on posture and proprioception should be integrated into music training sessions to reduce injury risk (Árnason, Briem & Árnason, 2018; Adamčák et al., 2025; Baadjou et al., 2018; Roos et al., 2024). Nevertheless, barriers to implementation remain significant, including lack of time, motivation, and institutional support (Ajidahun et al., 2019). Therefore, a multidisciplinary approach actively involving different

specialized professionals, educators and performers is essential to effectively address these issues.

Taken together, the results of this study support the growing body of literature advocating for preventive interventions that address not only ergonomic factors but also sensorimotor function in the health management of professional musicians, thus reinforcing the need for a comprehensive and integrative evidence-based approach. Unlike previous research predominantly based on self-reported data or isolated assessments, the present multidimensional framework might provide a more detailed characterization of the mechanisms underlying PRMDs. In particular, the integration of objective biofeedback-based measurements with subjective and functional evaluations may represent a promising methodological advancement in this field, concretely addressing some of the limitations highlighted by recent reviews (Stanhope et al., 2019; Rousseau et al., 2023). However, some limitations should be acknowledged. The relatively small sample size and the unbalanced distribution among instrumental groups may limit the generalizability of the findings. Moreover, the cross-sectional study design does not allow causal relationships to be established between exposure variables and outcomes. Future investigations should therefore adopt longitudinal designs and larger samples to better investigate the temporal evolution of sensorimotor adaptations and their role in the onset and prevention of PRMDs. In particular, it would be valuable to determine whether structured proprioceptive and postural re-education programs might positively modify PAS scores, improve the Libra-derived stability index, and reduce the incidence and severity of PRMDs over time. Applying a kinesiological perspective, a dose-response approach would be useful to provide standardized and instrument-tailored recommendations.

CONCLUSIONS

In conclusion, our investigation supports the concept that PRMDs in musicians emerge from an interplay between biomechanical constraints, reduced postural awareness, and lack of sensorimotor education. Accordingly, tailored interventions should incorporate proprioceptive training, postural re-education, and body awareness techniques, as well as structured physical conditioning programs designed, led and monitored by specialized kinesiologists (Baadjou et al., 2018; Carretti et al., 2024a; Roos et al., 2024). Regular integration of health education into music curricula may enhance early recognition of risk factors and promote long-term musculoskeletal health in this vulnerable target of individuals (Adamčák et al., 2025; Árnason, Briem & Árnason, 2018). While addressing barriers to implementation remains a critical challenge, both at institutional and individual levels (Ajidahun et al., 2019), concretely fostering such holistic approach might represent a key step toward the development of targeted and enjoyable protocols to preserve overall health, artistic performance, and career sustainability in this highly specialized population.

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AUTHOR CONTRIBUTIONS

GC: Conceptualization, Methodology, Formal analysis, Data curation, Investigation, Visualization, Writing – original draft, Writing – review & editing. TL: Methodology, Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing. MM (Mirko Manetti): Formal analysis, Data curation, Writing – original draft, Writing – review & editing. MM (Mirca Marini): Conceptualization, Methodology, Formal analysis, Data curation, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Embedding virtual tools and innovative approaches in neuroanatomy teachings: A single center pilot and feasibility experience

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Abstract. A solid knowledge of neuroanatomy is essential given the rapidly increasing use of neuroimaging in clinical practice. As a result, developing innovative and engaging methods to support students throughout the learning process has become a central topic of interest. Implementing digital tools and interactive approaches represents an elegant and promising solution. At our University, the international degree program in Medicine and Surgery (IMS)—taught entirely in English and welcoming students from all over the world—was established in the 2017–2018 academic year. IMS follows a specific framework for teaching human anatomy: during the first year, students attend a course entitled *Fundamentals of Human Morphology*, which provides the foundational knowledge needed for their training. In subsequent years, anatomy is revisited through the so-called *vertical tracks*, in which it is presented from a clinically oriented perspective. Moreover, the number of IMS students is smaller than in the Italian course ($n < 50$), making IMS an ideal environment in which to introduce and implement innovative strategies relying heavily on virtual dissections, gamification, and flipped-classroom approaches in a pilot and feasibility phase. In this paper, we describe the implementation of the advanced neuroanatomy course, designed to achieve a seamless integration of frontal lectures and interactive teaching methods. Survey results from 30 voluntary participants demonstrated high satisfaction, with median scores of 9.5/10 for the use of the virtual dissection table compared to textbooks, and 10/10 for the gamification tournament. Knowledge retention improved from a mean baseline score of 64% correct answers in the pre-test to over 85% of students answering all questions correctly in the post-test, confirming the feasibility and potential efficacy of this interactive curriculum.

Keywords: virtual dissection, innovative teaching methods, gamification, flipped classroom, human anatomy.

INTRODUCTION

A comprehensive and rigorous understanding of neuroanatomy is essential for the management of disorders involving both the central and peripheral nervous system. Such knowledge is fundamental for the correct inter-

pretation of the neurological examination and for the initiation of an appropriate diagnostic workup, which relies extensively on neuroanatomically based investigations including computed tomography (CT), magnetic resonance imaging (MRI), nuclear medicine techniques, and ultrasound (US) [1-5], as well as neurophysiological assessment [6]. Despite often being regarded as a domain primarily relevant to specialists in neuro-psychiatric disciplines – such as neurology, neurosurgery, psychiatry, child neurology/psychiatry, neuroradiology, and physiotherapy – neuroanatomy now plays a crucial role across a broader spectrum of healthcare professionals.

In the emergency setting, recent advancements in the treatment of acute ischemic stroke [7-10] and the incorporation of endovascular therapy into standard clinical practice [11] have reshaped organizational models [11,12], thereby requiring multidisciplinary teams to possess adequate neuroanatomical competence. Similarly, US has gained prominence beyond its traditional application in the diagnosis of peripheral neuropathies [3,4], becoming an essential tool for guiding procedures such as regional anesthesia and pain management [13-16]. These developments collectively underscore how neuroanatomy has evolved into an indispensable component of contemporary clinical practice, extending well beyond the boundaries of classical neuro specialties.

Stemming from these considerations, we introduced a tentative approach aimed at developing novel strategies to empower students and actively engage them in the learning process, with the goal of fostering a solid, clinically oriented neuroanatomical foundation that would remain stable over time. At our University, the international degree program in Medicine and Surgery (IMS) is structured in a way that is particularly suitable for this purpose. During the first year, students attend the *Fundamentals of Human Morphology* course, which provides the essential basis for their anatomical training. In the following years, anatomy is revisited within the so-called *vertical tracks*, where it is taught from a clinically oriented perspective. As an example, we describe here the implementation of this framework in the context of neuroanatomy teaching.

The IMS course was established in the 2017–2018 academic year (AY) and was structured differently from the Italian course (IC), not only because all classes are delivered in English – thus accommodating an international cohort of students from around the world – but also in its overall educational design.

During the first two years, the curriculum is intended to build a solid foundation in the basic (preclinical) disciplines, including human anatomy. To this end, as mentioned, students attend the course *Fundamentals of Human Morphology*, which provides a comprehensive introduction to human anatomy across all organs, systems, and regions. However, this represents only the initial phase of their training.

In the subsequent years, teaching is organized into the so-called *vertical tracks*, a system of integrated courses in which each medical topic is explored in depth, beginning with clinically oriented anatomy, physiology, and pathology, and progressing toward semiotics, diagnostics, and the nosographic classification and treatment of diseases related to that system.

With regard to neuroanatomy, students in the fourth year (of the six-year program) attend an advanced course embedded within the *Neuroscience vertical track*. This course is designed to establish a rapid and explicit link with clinical applications. By this stage, students already possess a foundational understanding of neuroanatomy, which they further consolidate to the point of integrating anatomical knowledge seamlessly into clinical reasoning.

Additionally, the relatively small class size – compared with the IC – makes IMS an ideal environment for introducing innovative, interactive teaching strategies. In 2017–2018 AY, the program offered 36 seats, a number that progressively increased to 48 seats by 2023–2024 AY.

Here, we describe how the advanced neuroanatomy course was progressively refined starting in 2021–2022 AY and subsequently consolidated into the format presented in this paper for the most recent academic year (2024–2025 AY, syllabus available here: Summary of Neuroanatomy I | e-Learning - UNIMIB; https://elearning.unimib.it/course/info.php?id=56249).

The primary objective was to assess the feasibility, student acceptance, and preliminary educational impact of a multifaceted, interactive neuroanatomy curriculum within an international medical program. While individual digital tools are increasingly common, the novelty of our approach lies in the seamless, structured integration of continuous virtual dissection, peer-to-peer gamification tournaments, and a flipped-classroom model, tightly aligned with clinical applications in a vertical-track curriculum

METHODS

All lessons for this course are delivered in the Anatomy Room, which is equipped with 3D plastic models and a virtual dissection tool, the Anatomage Table™ (Anatomage Inc., Santa Clara, California, US). This device provides 3D reconstructions of five segmented cadavers, enabling hands-on virtual dissections. It also includes its own case library and allows the upload of Digital Imag-

ing and Communication in Medicine (DICOM) data, which can be rendered with photorealistic filters. In addition, the software supports the upload and easy visualization of DICOM datasets originating from users' own clinical practice, facilitating in-depth discussion of real clinical cases with strong anatomical relevance.

Furthermore, each student received an individual license (purchased by the University) for the companion e-book/platform associated with the Anatomage system, Anatomage Lessons™. Paper-based exercises (primarily structure coloring and labelling activities) were also provided.

During the 2024-2025 AY, each lesson was delivered in a 4-hour session combining both lecture-based and interactive activities. Students (n = 36) were divided into six teams of six members each, with team names chosen by the students themselves. These groups collaborated during the interactive breakout sessions.

At the beginning of the very first lesson, students anonymously completed a self-assessment multiple-choice questionnaire to gauge how much knowledge they had retained from the first-year course. The test was accessed anonymously via mobile phone through a Google form linked to a QR code.

At the start of all subsequent lessons, the class was divided into groups and each session opened with an assignment. The Anatomage Table™ quiz mode enabled the creation of a tournament: each group, one at a time, performed an 8-minute dissection exercise, generating a score that was recorded. Each quiz was designed to review and reinforce the topic covered in the previous lesson. While one group was engaged in this activity, the others worked collaboratively on exercises using the Anatomage Lessons™ platform and paper-based materials.

The lecture-based portion of the lesson then followed. Topics were presented through a combination of virtual dissections and minimal use of traditional slide decks, which were reserved primarily for diagrams and summaries. After each topic was introduced, students again worked in teams: one team at the Table and the others completing paper-based or Anatomage Lessons™ activities.

Considering the available literature on the topic[17-20], a gamification approach was tentatively integrated into the course. During the final lesson, the Table's quiz mode was used once again to assess all teams on the entire syllabus. By pooling the scores accumulated across all lessons, one team was ultimately declared the winner of the tournament. Finally, students were given the opportunity to retake the self-assessment multiple-choice questionnaire.

Students who attended the neuroanatomy course in the 2024–2025 AY were the same cohort that had received human anatomy instruction exclusively online in 2019–2020 AY due to the COVID-19 pandemic. This made them an ideal group for evaluating whether a more engaging, interactive approach would be perceived as beneficial.

At the end of the course, students were invited to anonymously rate the activities conducted throughout the program using an ad hoc evaluation questionnaire developed via a Google form accessed via a QR code; completing the survey was not mandatory. The evaluation followed the standard 0–10 grading system used by our university for assessing students' opinions on teaching activities (https://opinionistudenti.unimib.it/valdid/): low (0-4), intermediate (5-7), and high score (8-10).

RESULTS

Thirty students voluntarily and anonimously provided their feedback. Figure 1 summarizes the perceived usefulness of the live dissections performed by the professor during the lecture-based portion of the lessons, showing overall highly satisfactory results.

Figure 2 presents students' ratings of the activities they felt were most empowering. The online materials – offered through *Anatomage Lessons*™ – were particularly appreciated, also supporting a flipped-classroom approach that is gaining increasing relevance in anatomy education. Since the platform was accessible at all times, students could review topics in advance, as all materials were made available from the very first lesson. All learning modalities (i.e., online materials, paper-based exercises, and the gamification approach) received more than satisfactory scores, although paper-based exercises were rated slightly lower overall.

The following median scores were recorded for the most relevant items:

a) use of the Table compared with a regular textbook: 9.5 (Q1: 8.25; Q3: 10);

b) importance of students directly using the Table: 9 (Q1: 8; Q3: 10);

c) use of Anatomage Lessons™: 9 (Q1: 8; Q3: 10);

d) completion of paper-based exercises: 6.5 (Q1: 4; Q3: 8);

e) Anatomage tournament integrated into the lessons: 10 (Q1: 8.25; Q3: 10).

To tentatively assess the effectiveness of the teaching method, performance on the preliminary multiple-choice test was compared with that on the final test. At the beginning of the course, students achieved

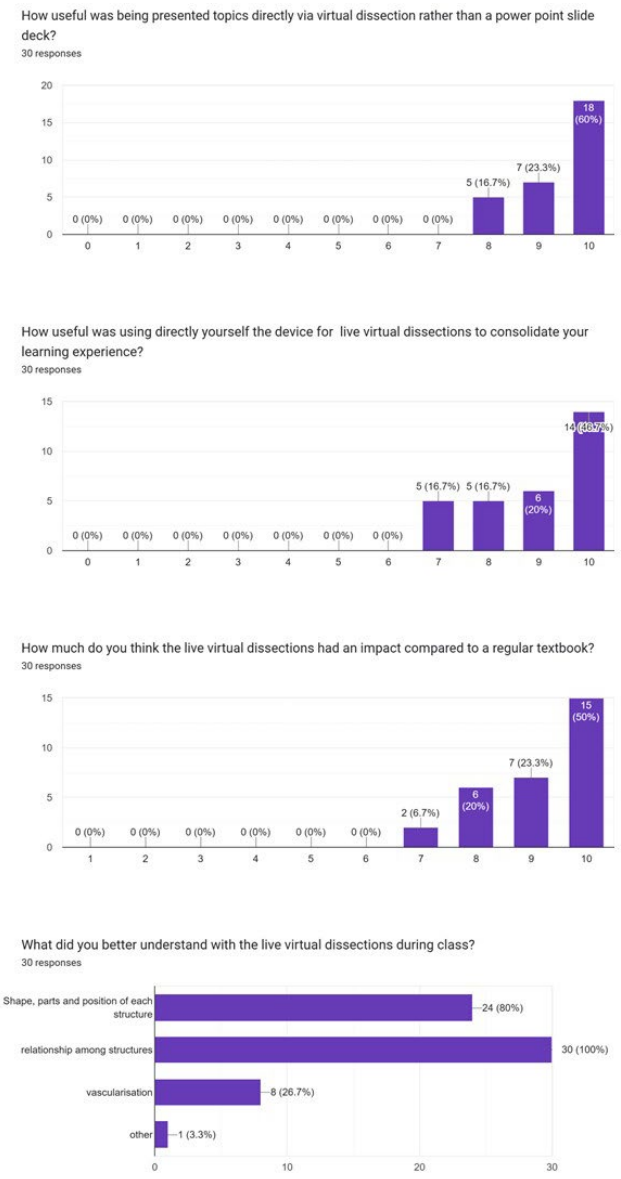

Figure 1. Rating of the usefulness of live virtual dissections during class. Scoring system: low (0-4), intermediate (5-7), and high score (8-10).

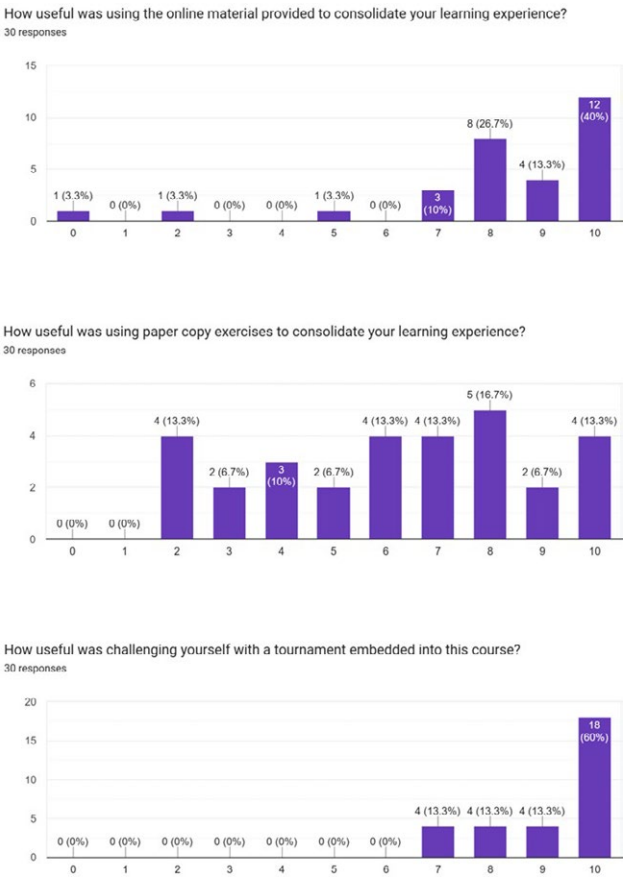

Figure 2. Rating of the usefulness of actions requiring students' active participation. Scoring system: low (0-4), intermediate (5-7), and high score (8-10).

a mean score of 64% correct answers overall, with only 25% of questions answered correctly by fewer than 45% of students. After completing the course, more than 85% of students answered all questions correctly; moreover, 25% of the questions received correct answers from the entire cohort, and even the question with the lowest success rate was answered correctly by 83% of students. It should be noted, however, that the same questions were used in both tests and, as these were not formal examinations, students may have had access to study materials on both occasions.

DISCUSSION

Our data, although preliminary and primarily intended as a feasibility assessment, were consistent with the published literature, which highlights the importance of virtual and 3D approaches in learning anatomy compared to traditional 2D tools[21-24]. Furthermore, the survey results were once again consistent with previously published approaches to anatomy teaching, highlighting both the relevance of gamification and the value of a student-centered learning environment[19,24-26].

The evaluation of our educational experience inevitably intersects with the global and ongoing debate regarding the role of traditional cadaveric dissection versus digital innovations in modern medical curricula. As recently highlighted in an interactive consensus feature in the *New England Journal of Medicine*[27], medical schools face critical policy decisions regarding whether to maintain resource-intensive body-bequest programs or fully embrace advanced technological learning tools.

Proponents of traditional dissection argue that working with human donor bodies provides irreplaceable, transformative opportunities, acting as a 'first clinical discipline' where students acquire emotional competencies, teamwork, and a profound awareness of anatomical variation. Conversely, a recent debate by Shastri and colleagues[28] emphasizes that modern undergraduate medical teaching has shifted toward multimodal, educationally principled curricular designs. They point out that traditional dissection is heavily resource-laden, requiring specialized laboratories and embalming chemicals, and is exceptionally time-intensive within an increasingly packed undergraduate medical curriculum; furthermore, they note that disproportionate student time is often spent removing fat or connective tissue rather than conceptualizing functional anatomy, whereas digital 3D anatomy tools and interactive applications facilitate inclusive, self-paced, and flexible learning with precise structural visualization.

In this context, advanced digital platforms provide an elegant, effective bridge. They allow students to engage with photorealistic 3D anatomical networks in ways that go beyond traditional methods, effectively preparing them for a healthcare environment increasingly dominated by radiologic imaging and digital interfaces. Rather than viewing these modalities as mutually exclusive, our pilot experience suggests that virtual tools can successfully act as powerful pedagogical enhancements that respect the tight time constraints of modern undergraduate training while actively empowering students through interactive, student-centered frameworks

Overall, this experience proved highly promising, and further developments are already underway. During the first year, one of the modules of the *Fundamentals of Human Morphology* course, *Regional Anatomy*, has already been delivered using an approach similar to the one described in this paper, starting from 2023–2024 AY. The same applies to a course on the female reproductive system for sixth-year students (implementation initiated in 2024–2025 AY) within the *Woman and Child* vertical track. Likewise, the *Anatomy of the Digestive System* course (part of the *Digestive Health* vertical track) for fifth-year students is currently being revised following the same framework, with implementation beginning in 2025–2026 AY.

In addition to updating the curricula of formal courses, we are also promoting a peer-to-peer tutoring program, whose positive impact was documented in a publication describing its pilot phase; the initiative was highly appreciated by students in both the IMS and IC degree programs[29]. This activity will be further expanded to include peer-to-peer tutors specifically trained to support younger students in the independent use of the Table, as a second Table is being made available in the library and will be accessible to all trained students through a dedicated booking system.

The approaches described above constitute a preliminary pilot phase during which the teaching methods were cautiously upgraded in response to students' emerging needs. A formal research protocol is being developed to systematically determine the effectiveness and long-term impact of this educational trajectory. Several limitations, in fact, must be acknowledged in this pilot study. First, it is a single-center experience with a relatively small sample size (n = 36, with 30 survey respondents), which may limit the generalizability of our findings to larger student cohorts or different institutional settings. Second, the study lacks a parallel control group (e.g., students undergoing traditional lectures only), preventing a definitive causal conclusion regarding the superior educational efficacy of the digital approach. Third, the evaluation of student satisfaction relies on self-reported outcomes, which are inherently subject to subjective bias. Lastly, because the pre- and post-tests utilized the identical multiple-choice questions in a non-formal examination environment, the observed performance improvement must be interpreted with caution, as it might partly reflect test familiarity or access to study resources rather than long-term knowledge retention. These limitations will be addressed in our upcoming formal research protocol involving a controlled, longitudinal evaluation.

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Dynamic tunnelling nanotube formation characterizes AML–Mesenchymal Stromal cell crosstalk in the Bone Marrow Microenvironment

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Abstract. Acute myeloid leukaemia (AML) is a rapidly progressing cancer that leads to the accumulation of immature blasts in the bone marrow (BM). The BM niche, in particular mesenchymal stromal cells (MSCs), supports and protects AML cells, leading to resistance to treatment and relapses. AML blasts actively interact with mesenchymal MSCs, reshaping the niche and engaging in direct cytoplasmic exchange through tunnelling nanotubes (TNTs). Using cocultures of MOLM-13 AML cells and MS-5 stromal cells labelled with distinct vital dyes, we observed the rapid formation of TNTs-like protrusions within the first hours of contact. Phalloidin staining confirmed their continuous F-actin structure, consistent with canonical TNT morphology. Notably, TNT frequency increased following treatment, indicating that therapeutic stress enhances AML–MSC crosstalk. These findings show that AML establishes fast, dynamic physical interactions with MSCs and that TNT formation is further stimulated by treatment, highlighting TNT-mediated crosstalk as a potential target to weaken stromal protection and improve therapeutic response.

Keywords: AML, BM microenvironment, tunnelling nanotubes, MSC.

INTRODUCTION

AML is a very aggressive and rapidly growing subtype of blood cancer. It is a genetically heterogeneous disease, in which haematopoietic stem cells undergo events of mutations, resulting in the deregulation of self-renewal, proliferation, and differentiation pathways. Such mutations occur in Hematopoietic Stem Cells (HSCs) and cause the transformation in Leukemic Stem Cells (LSCs), which arrest differentiation and lead to the accumulation of immature myeloid blasts in the bone marrow, impeding the production of functional blood cells (Kayser et al., 2018).

Research has demonstrated that the BM microenvironment plays a dual role, both protective and supportive, for AML cells, creating a specialized niche. The BM niche is characterized by complex populations of cells,

including various subpopulations of MSCs, stromal cells, endothelial cells (ECs) and osteolineage cells (OLCs). Moreover, new inferred osteolineage differentiation trajectories, and distinctions among Lepr, Nestin, and NG2-expressing HSC niche populations are defined. A recent study used single-cell RNA sequencing to identify up to six major cell types with 17 distinct cell subsets including sinusoidal, arteriolar and arterial ECs, several fibroblast subtypes, pericytes, chondroblasts and OLCs. However, AML disrupts the stromal landscape to their favour, particularly by impairing mesenchymal and osteogenic differentiation and reducing the expression of key hematopoietic regulatory factors. These changes create a less supportive microenvironment for normal haematopoiesis, giving malignant cells a competitive advantage (Baccin et al., 2020; Baryawno et al., 2019).

Two models for the involvement of the BM niche in AML development have been suggested. One model suggests that leukemic cells can remodel the BM niche to their advantage, creating a microenvironment that promotes AML progression. The other suggests that genetic alterations within the BM niche cells themselves initiate and contribute to the development of myeloid malignancies. Both models are supported by evidence of complex crosstalk pathways and self-reinforcing feedback loops (Schepers et al., 2015).

Indeed, such protective role of the BM niche towards AML cells has been strongly demonstrated. Particularly mesenchymal stem cells activate several pathways through direct contact or soluble factors which lead to the protection of AML cells from treatment. The BM niche is a key driver of cell adhesion-mediated drug resistance. In this process, leukemic cells exploit physical interactions with components of the BM microenvironment, including MSCs, ECs, and osteolineage cells, to evade apoptosis and withstand chemotherapy. Through adhesion molecules on their surface, leukemic cells activate signalling pathways that promote the establishment of a protective, pro-leukemic niche. At the same time, soluble mediators such as cytokines and chemokines, most notably CXCL12, further contribute to therapy resistance by reinforcing survival and resistance (Allert et al., 2024) (Fig. 1).

Among the different genetic alterations found in AML, mutations in the FMS-like tyrosine kinase 3 (FLT3) are the most common and with worse prognosis. Such mutations can occur as internal tandem duplication (FLT3-ITD) in the juxtamembrane domain or as a point mutation in the tyrosine kinase domain (FTL3-TKD), causing constitutive activation of the receptor and its downstream signalling pathways. Notably, FLT3-ITD+ AML cells undergo intrinsic endoplasmic reticulum (ER) stress due to the accumulation of FLT3-ITD misfolded protein in the ER (Takahashi S., 2020).

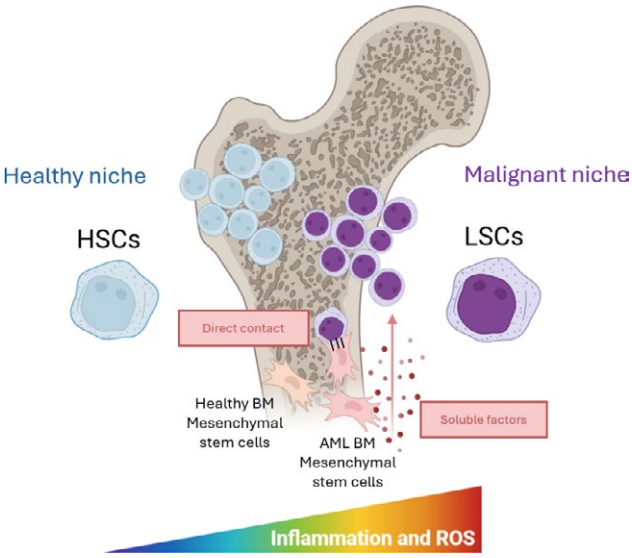

Figure 1. Schematic representation of the bone marrow niche and its progressive transformation from a healthy bone marrow niche into a malignant niche in acute myeloid leukaemia (AML). On the left, healthy hematopoietic stem cells (HSCs) reside in a normal microenvironment maintained by bone marrow mesenchymal stem cells (MSCs). On the right, leukemic stem cells (LSCs) remodel the stromal compartment, generating an AML-supportive niche characterized by altered MSC function that promotes leukemic survival and clonal expansion. LSCs–MSCs communication occurs through direct cell–cell interactions and soluble factors like cytokines and chemokines, altogether activating pro-leukemic signalling pathways. The gradient at the bottom depicts the increasing levels of inflammation and reactive oxygen species (ROS) that accompany niche malignant transformation, contributing to metabolic rewiring, stress adaptation, and AML progression. Figure created with BioRender.com.

A recent study demonstrated how Retinoic acid and proteotoxic stress induce AML cell death overcoming stromal cell protection. Indeed, the combination of Retinoic Acid (R), Bortezomib (B) and Arsenic Trioxide (A) resulted strongly cytotoxic on FLT3-ITD+ AML cell lines and primary blasts isolated from patients due to ER homeostasis imbalance and generation of oxidative stress. However, as mentioned, the BM niche exerts a protective mechanism against treatment *in vivo*, rendering AML cells completely resistant to the RBA combination. For this reason, it was exploited a coculture system using a FLT3-ITD+ AML cell line (MOLM-13) and a murine stromal cell line (MS-5) to reproduce a more relatable BM microenvironment. As expected, MS-5 protected AML cells from the RBA combination. To overcome the protective mechanism, high doses of ascorbic acid (ASC) were added as an adjuvant

to induce even more oxidative stress. The RBA + ASC resulted efficient in eliminating AML cells in vitro and in vivo (Liccardo et al., 2023).

Such evidence highlights the complex and necessary crosstalk between AML cells and MSCs, reinforcing the need to comprehend the molecular basis and interactions of the communications, rendering inevitable the study of AML as a complex and dynamic system involving more than one cell type. As previously discussed, there are many different strategies and pathways that MSCs activate to protect and support AML cells particularly during treatment. One of these is by enhancing antioxidant defences.

LSCs retain several properties of normal hematopoietic stem cells, yet their metabolism is profoundly reprogrammed to sustain the elevated energetic and biosynthetic demands of AML cells and notably, chemoresistant populations depend on mitochondrial oxidative phosphorylation (OxPhos) to meet their metabolic needs. Recent studies have shown that MSCs can transfer mitochondria to AML cells in vitro, supplying them with additional bioenergetic capacity. This transfer is further amplified following chemotherapy and is thought to promote resistance by preventing mitochondrial depolarization. Although AML cells already display elevated reactive oxygen species (ROS), leukemic stem cells maintain high yet non-toxic ROS levels through upregulated antioxidant pathways. In parallel, BMSC-driven increases in OXPHOS, TCA cycle activity, and glutathione (GSH)-mediated antioxidant defenses enable AML cells to meet their metabolic demands and withstand chemotherapy-induced stress. Together, these findings suggest that mitochondrial acquisition from the microenvironment is a key component of AML metabolic adaptation and therapy resistance (Forte et al., 2020).

In support of this, mitochondrial transfer has been experimentally demonstrated between the murine stromal cell line MS-5 and multiple AML cell lines. This process was quantified by flow cytometry, in which MS-5 cells were pre-labelled with MitoTracker and the subsequent uptake of MitoTracker-positive mitochondria by AML cells was measured. The phenomenon was independently validated by qPCR detection of mouse-derived mtDNA within human AML cells, providing molecular evidence of cross-species mitochondrial acquisition and reinforcing the functional relevance of stromal-to-leukemia mitochondrial transfer (Moschoi et al., 2016).

In fact, mitochondrial transfer has emerged as a relevant phenomenon within the tumour microenvironment, occurring not only among tumour cells but also between tumour cells and cells from the microenvironment including stromal cells, immune cells, endothelial cells, platelets, and even homologous cells. In most contexts, cancer cells acquire mitochondria from surrounding cells to enhance invasion, metastasis, chemotherapy resistance, and immune evasion (Brestoff, J. R. 2025).

Among the various mechanisms described, TNTs represent a common mechanism for intercellular mitochondrial transfer. TNTs are thin (50–1500 nm in diameter and 5–200 μm in length, with some reaching 700 nm thickness), F-actin–based membrane tubes that physically connect the plasma membranes of two cells, creating a direct bridge between the cells allowing exchange of cytoplasmic material including mitochondria. Two major models of TNT formation have been proposed. In the actin-driven mechanism, a cell extends an actin-rich protrusion that fuses with a neighbouring cell, enabling TNT formation independently of cell motility. In contrast, the cell-displacement mechanism occurs when two adjacent cells migrate in opposite directions, stretching membrane connections into TNTs; this process is highly dependent on cell movement (Guan et al., 2024).

Within the AML bone marrow niche, stromal–leukemic cell interactions create a supportive environment that promotes leukemic cell survival. AML blasts rely heavily on OxPhos and continuously adapt to fluctuations in nutrient and oxygen availability within the BM niche. Recent studies have shown that reactive oxygen species (ROS) stimulate MSCs to transfer mitochondria to leukaemia cells via TNTs, particularly under stress or chemotherapy. The acquisition of functional mitochondria increases mitochondrial respiration and ATP production in recipient AML cells, thus enhancing overall functional activity (Saito et al., 2021).

Taken together, these findings highlight the strong dependence of AML cells on MSCs, particularly under therapeutic stress. In this study we show that such crosstalk is rapid and dynamic, occurring in the first hours of direct contact and increases upon therapeutic stress.

METHODS

Cell culture

The human AML cell line MOLM-13 was cultured in RPMI 1640 medium supplemented with 1% penicillin/streptomycin and 7% FBS. The murine BMSC line MS-5 was cultured in MEMα with 1% penicillin/streptomycin, 20% FBS and 100 μM β-mercaptoethanol. All the cultures were maintained at 37°C in a humified atmosphere containing 5% CO_2.

Co-Cultures

50,000 MS-5 cells per well in an Ibidi µ-Slide 8 Well high ibiTreat (cat# 80806, Ibidi, Gräfelfing, Munich, Germany). After 72 hours, when cells reached approximately 80% confluency, the medium was replaced with 1 mL of RPMI 7% FBS 1%penstrep. The following day, MOLM13 cells were added at a concentration of 70,000 cells/mL, followed by treatment with 5-azacitidine (A) 100 nM and Venetoclax (V) 10 nM.

Live cell stain

MS5 cells were incubated with 1.67 µL of DiD in 500 µL of MEM Alpha medium (without serum) for 20 minutes at 37 °C in an incubator. After incubation, cells were washed twice with MEM Alpha containing serum to inactivate the dye, then resuspended and plated at 50,000 cells per well in an Ibidi µ-Slide 8 Well high ibiTreat (cat# 80806, Ibidi, Gräfelfing, Munich, Germany).

After 24 hours, MOLM13 cells were stained with DiI (1 µL/mL) for 20 minutes at 37 °C in serum-free RPMI medium, followed by three washes in RPMI supplemented with 7% FBS. The medium from the MSCs was then removed, and the DiI-labelled MOLM13 cells were added to the co-culture together with the respective treatments. After 6 hours, co-cultures (MOLM13-DiI / MS5-DiD) were analysed by confocal microscopy using a live-cell imaging chamber equipped with a heater and humidifier, maintaining stable conditions of 37 °C, 5–7% CO_2, and high humidity to preserve cell viability and physiological behaviour during imaging. DiD and DiI dyes were purchased from Thermo-Fisher Scientific (Vybrant™ Multicolor Cell-Labeling Kit, Cat# V22889).

For phalloidin staining, after fixation in 4% PFA and permeabilization with 0.1% TX100, cells were incubated with Rhodamine Phalloidin (R415, Thermo-Fisher Scientific) at a 1:50 dilution in 1% BSA-PBS for 30 minutes at room temperature. Nuclei were counterstained with DAPI (dilution 1:1000) for 8 minutes. Images were acquired and analysed by confocal microscopy by a Zeiss LSM 900 with Airyscan 2, equipped with ZEN 3.2 Blue Edition software.

TNTs quantification

TNTs were quantified based on established morphological criteria. Structures were classified as TNTs when they appeared as thin protrusions, contained F-actin as confirmed by phalloidin staining. For each biological replicate, 100 microscopic fields were analysed. Image evaluation was performed in a blinded manner to minimize observer bias during TNT identification.

RESULTS

To highlight the interaction between AML cells and MSCs by TNTs, we differentially stained the murine stromal cell line MS-5 with the vital dye DiD (red) in coculture with the human AML cell line MOLM-13, stained with the vital dye DiI (yellow). Confocal microscopy revealed the presence of thin yellow nanotube-like structures extending from AML cells toward MS-5 cells, demonstrating direct crosstalk. These structures were detectable within the first hours of direct co-culture (Fig. 2A–B), revealing the rapid and dynamic nature of AML–MSCs communication.

To confirm that the observed structures were consistent with TNTs features, both MS-5 and MOLM-13 cells were stained with rhodamine phalloidin to visualize F-actin. The connecting protrusion showed a continuous actin structure, in line with the canonical description of TNTs (Fig. 2C). Finally, given the central role of the bone marrow niche in promoting AML survival and therapy resistance, we assessed whether treatment influenced TNTs formation. Indeed, we observed an increase in TNTs frequency upon Azacytidine and Venetoclax (Aza-Ven) treatment, indicating that AML–MSCs communication is further enhanced under therapeutic pressure (Fig. 2D).

DISCUSSION

AML is a heterogeneous and rapidly progressing blood cancer originating in the bone marrow. Many studies have shown that AML cells rely heavily on mesenchymal stromal cells for protection, metabolic support, and resistance to therapy. MSCs, endothelial cells, and osteolineage cells together create a pro-leukemic niche that impairs normal differentiation and favours leukemic expansion. Recent work has also highlighted how AML cells use tunnelling nanotubes and mitochondrial transfer to strengthen this interaction, especially under stress or chemotherapy. This makes the AML–MSCs relationship a key contributor to treatment resistance and an important target for new therapeutic strategies.

Our findings demonstrate that AML–MSCs communication is an early, rapid, and highly dynamic process, established within the first hours of direct contact and intensified under therapeutic pressure. By combin-

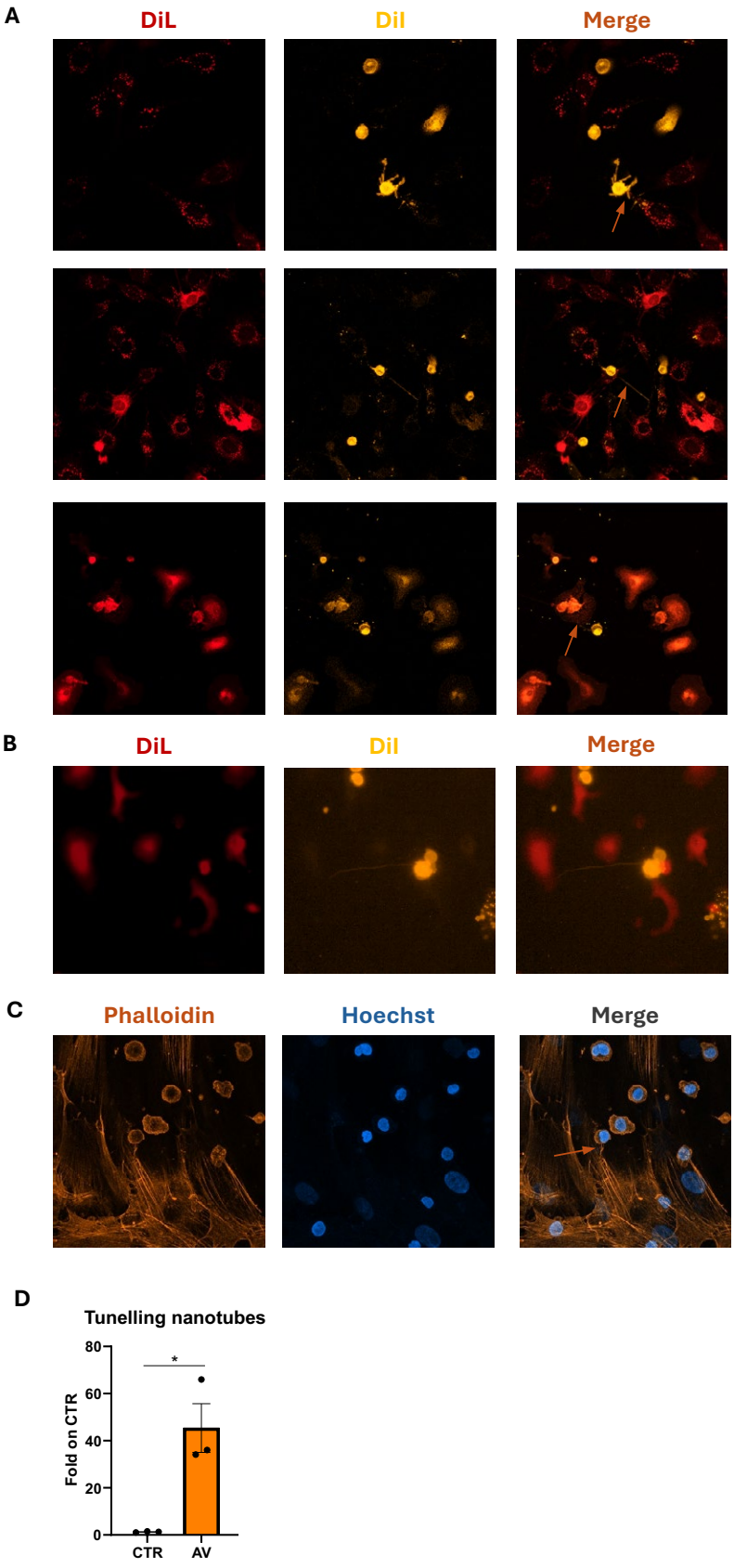

Figure 2. A) Murine stromal cells (MS-5), labelled in red with the lipophilic vital dye DiD, were cocultured with human AML cells (MOLM-13), labelled in yellow with DiI. Cells were independently labelled with lipophilic vital membrane dyes prior to coculture. Orange arrows indicate TNTs connecting AML cells with MS-5 cells. Images were acquired by confocal microscopy after 2 hours of coculture. B) Wide-field image showing a DiI-labelled MOLM-13 cell extending a TNT towards a DiD-labelled MS-5 cell after 2 hours of coculture. C) MS-5 and MOLM-13 cells were fixed and stained with phalloidin to visualize F-actin organization within TNTs and cell bodies. Nuclei were counterstained with Hoechst (blue). This staining confirms the actin composition of TNTs providing structural validation of the intercellular protrusions observed in A and B. D) Quantification of TNTs formed between MOLM-13 AML cells and MS-5 stromal cells after treatment with Aza-Ven. (AV). (Each dot represents a biological replicate from independent experiments, bar represents the mean ± S.E.M; n=3, Unpaired t-test *P= 0,0129).

ing differential vital-dye labelling with confocal imaging, we show that AML blasts extend TNTs toward bone marrow stromal cells, forming thin, actin-rich cytoplasmic bridges that enable direct intercellular exchange. Although phalloidin staining confirms the presence of F-actin–containing protrusions, we acknowledge that TNTs identification is based primarily on morphological criteria. Nonetheless, the observed thin, actin-rich structures connecting distant cells are fully consistent with the canonical description of tunnelling nanotubes.

Importantly, we observed that TNTs formation is not static, but rather dynamic and amplified in response to treatment, suggesting that therapeutic stress increases the physical and metabolic crosstalk between AML cells and MSCs. This aligns with emerging evidence that the bone marrow niche actively contributes to leukemic resistance by enhancing metabolic support, antioxidant defences, and mitochondrial transfer during therapy.

Together, these results reinforce the concept that AML progression and therapy resistance cannot be understood solely through leukaemia-intrinsic mechanisms. Instead, AML must be considered as a multicellular system, in which stromal cells support and protect AML cells, enabling leukemic blasts to withstand cytotoxic stress. The rapid induction and treatment-driven amplification of TNT-mediated communication highlights TNTs as a functionally relevant component of AML–MSCs crosstalk and a potential therapeutic vulnerability. While we demonstrate that TNTs formation is rapidly induced and enhanced by treatment, future studies will be required to assess whether these protrusions support functional transfer of mitochondria or cytoplasmic material, thereby establishing a direct functional connection between TNTs formation and AML survival mechanisms within the stromal niche.

Overall, this study underscores the need for therapeutic strategies to focus not only on leukemic cells alone but on the entire niche, limiting TNT-mediated material exchange, and weakening the protective influence of the bone BM microenvironment. Targeting these microenvironment-dependent survival pathways may enhance the efficacy of existing treatments and improve outcomes for AML patients.

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Corrigendum of

Features of intestinal lesions in the clinical course of Inflammatory Bowel Diseases

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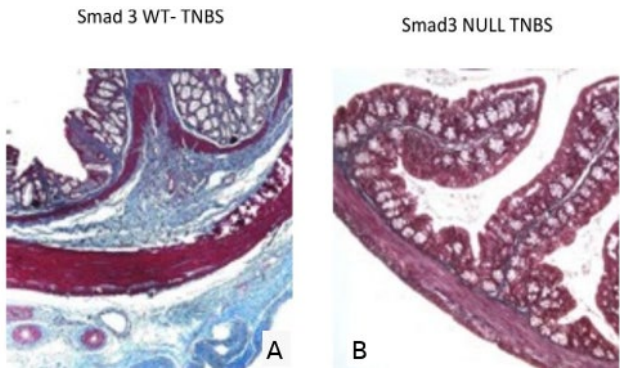

Figure 1. Following TNBS treatment, colon from Smad3 WT mice showed marked changes due to abnormal deposition of connective tissue in the serosa and to a lesser extent in lamina propria and submucosa (A). Whereas the colonic wall of Null mice appeared normal (B). Masson Thrichromic 4X.

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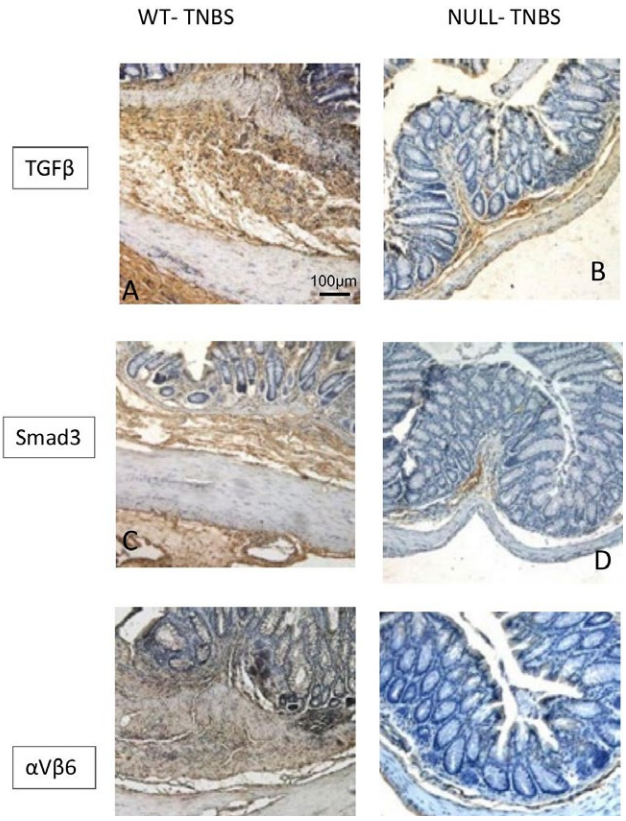

Figura 2. TGFβ, Smad3 and αVβ6 immunostaining (A,C,E respectively) were increased in colonic submucosa and serosa of Smad3 WT TNBS-treated mice compared to that of Smad3 null mice (B, D, F respectively). Immunohistochemistry, 10X.

The Authors underline that microphotograph related to Smad3 WT-TNBS was previously published in in Eur J Histochemistry, 2013 Dec 4;57(4):e40, https://doi.org/10.4081/ejh.2013.e40.

The image duplications were the result of an honest and unintentional error.

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