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New horizons in reconstructing past human behavior: Introducing the "Tübingen University Validated Entheses-based Reconstruction of Activity" method

Fotios Alexandros Karakostis[1] | Katerina Harvati[1,2]

[1]DFG (Deutsche Forschungsgemeinschaft) Center for Advanced Studies "Words, Bones, Genes, Tools," Eberhard Karls University of Tübingen, Tübingen, Germany

[2]Paleoanthropology, Senckenberg Centre for Human Evolution and Palaeoenvironment, Institute of Archaeological Sciences, University of Tübingen, Tübingen, Germany

Correspondence
Fotios Alexandros Karakostis and Katerina Harvati, DFG (Deutsche Forschungsgemeinschaft) Center for Advanced Studies "Words, Bones, Genes, Tools," Eberhard Karls University of Tübingen, Ruemelinstrasse 23, D-72070, Tübingen, Germany.
Email: fotios-alexandros.karakostis@uni-tuebingen.de (F. A. K.) and katerina.harvati@ifu.uni-tuebingen.de (K. H.)

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Abstract

An accurate reconstruction of habitual activities in past populations and extinct hominin species is a paramount goal of paleoanthropological research, as it can elucidate the evolution of human behavior and the relationship between culture and biology. Variation in muscle attachment (entheseal) morphology has been considered an indicator of habitual activity, and many attempts have been made to use it for this purpose. However, its interpretation remains equivocal due to methodological shortcomings and a paucity of supportive experimental data. Through a series of studies, we have introduced a novel and precise methodology that focuses on reconstructing muscle synergies based on three-dimensional and multivariate analyses among entheses. This approach was validated using uniquely documented anthropological samples, experimental animal studies, histological observations, and geometric morphometrics. Here, we detail, synthesize, and critically discuss the findings of these studies, which overall point to the great potential of entheses in elucidating aspects of past human behavior.

KEYWORDS

biocultural evolution, bone remodeling, habitual activity, hominin behavior, musculoskeletal stress, occupational stress

1 | INTRODUCTION

Muscle attachment sites (i.e., "entheses") are the areas of the bone where muscles, tendons, or ligaments attach.[1] They represent the only direct evidence of the musculotendinous, soft tissue system on skeletal remains. As such, they are commonly thought to broadly reflect muscular activity and have been used for reconstructing habitual physical activity among past human populations and extinct hominin species.[2–4] The methods of entheseal analysis can broadly be divided into those relying on observational scoring systems describing robusticity or potentially pathological entheseal changes (EC) of the surface on the one hand[3–7]; and those quantifying entire entheseal size or shape based on three-dimensional (3D) surface models on the other.[8–12] The interpretations of the latter approaches typically rely on the assumption that entheseal morphology may vary with changes of biomechanical load.[2,13,14] However, most visual scoring systems for analyzing these activity markers have often been criticized for a lack of precision and statistical rigor.[15,16] At the same time, several studies focusing on entheseal 3D morphology in laboratory animals and human cadavers have found no evidence to support the hypothesized functional character of entheses,[12,17–19] questioning their reliability in reconstructing past behavior.

In this framework, we recently put forth a new and repeatable methodology for analyzing entheses based on multivariate analyses targeting the reconstruction of muscle synergy groups.[10] As our approach relies on 3D surface area measurements of entheses without potential pathological lesions (i.e., distinctive osteophytic or osteolytic lesions),[10] it can be considered more comparable to visual scoring techniques focusing on entheseal robusticity, rather than those incorporating potentially pathological ECs.[20,21] Here, we conduct a critical review of the interdisciplinary body of literature that validated this 3D approach on the basis of anthropological samples with uniquely detailed occupational documentation[22] as well as two blindly-conducted experimental studies on animals.[23,24] These results are discussed together with our relevant findings from histological research on human cadaveric material[25] and 3D geometric morphometric applications for analyzing muscle attachment form.[11] Taken together, our synthesis supports the new approach's efficiency as an important tool for reconstructing past human behavior,[26] while also describing its limitations and potential pathways for improvement in the future. We term this new methodology the Tübingen University Validated Entheses-based Reconstruction of Activity (hereafter V.E.R.A.).

1.1 | Types of entheses and biomechanical implications

Muscle attachment sites are broadly categorized as fibrous or fibrocartilaginous, depending on the nature of their insertion to the bone.[1] In fibrous attachments, the muscle/tendon or ligament attaches directly onto the periosteum, which is itself attached to the bone via Sharpey fibers.[27,28] Fibrocartilaginous entheses, on the other hand, describe a gradient structure of attachment involving four transition zones: tendon, uncalcified fibrocartilage, calcified fibrocartilage, and bone.[1,27,29,30] Some studies of EC scores analyze fibrous and fibrocartilaginous entheses together.[20] However, most researchers consider fibrous entheses less useful for reconstructing physical activity, mainly due to the poor understanding of their reaction to biomechanical stress, lower measurement repeatability, weak correlation with occupational types, lower level of bilateral asymmetry in the upper limbs, and higher correlation with body size proxies.[5,6,31–34] This viewpoint is also supported by histological research suggesting that, in fibrous entheses (typically along the metaphysis or diaphysis of long bones), biomechanical forces seem to be dissipated over a wider area of attachment.[17,27] By contrast, in fibrocartilaginous entheses (typically at apophyses or epiphyses of long bones), all biomechanical stress is concentrated on to relatively smaller attachment surfaces, potentially leading to higher stress energy density and greater deformations of the comparatively thin cortical layer within these areas.[1,17,29] Nonetheless, it should be emphasized that recent histological research showed that fibrous and fibrocartilaginous attachments may co-exist in the same enthesis,[27,35,36] suggesting that the nature of muscle attachment to bone is much more complex than previously believed.

The anatomical area where an enthesis is located is also considered important, given that the nature and intensity of the biomechanical forces at play are bound to differ across the skeleton. As a result, there is substantial variation among entheses both in gross morphology and micro-architecture.[5,25,30] From a biomechanical point of view, entheseal variability has been suggested to be broadly associated with an interaction between body weight (compressive) and muscle tendon (tensile) forces, which substantially vary across anatomical regions.[25,30,37,38] In this regard, the hand shares the smallest proportion of body weight in the human skeleton,[39] suggesting that perhaps the effects of muscle recruitment on entheses may possibly be greater in hand bones compared to elements from high-weight-bearing anatomical areas.[25] Based on this concept, as well as on the fundamental role of manual tasks in human daily activities and subsistence strategies,[40] most of our research to date has focused on the entheses of the human hand.

2 | TESTING THE PRECISION OF 3D ENTHESEAL MEASUREMENTS

Multiple recent studies attempted to analyze entheses using measurements in three dimensions.[8–12,17,19,22–24,26,41] Nevertheless, the issue of measuring repeatability, which was directly raised by Noldner and Edgar,[8] was not adequately addressed in most of these previous works. Essentially, many of these studies did not mention a precision test, while some referred to unpublished intra-observer tests without, however, presenting the results or describing the criteria for delineating entheseal areas on the bones.[17,41] In contrast, Williams-Hatala et al.[12] performed precision tests on human hand entheses and reported low intra- and inter-observer repeatability error (reported mean inter-observer error: 4%), but did not outline the process, criteria, or tools used for determining the exact borders of entheses on the bone surface.

In 2016,[10] the first presentation of our method was accompanied by thorough intra- and inter- observer repeatability tests, which indicated a mean error rate below 1%. Based on our method, the process of delineating the exact borders of entheses on the bone surface relies on three criteria (i.e., elevation, complexity, and coloration) and the assistance of 3D imaging filters available in the software Meshlab (CNC Inc., Rome).[10,23] The exact 3D imaging filters used for our approach were also further described in our later publications on animal experimental data (for a detailed outline, see below Box 1 and Figure 1).[23,24] The most important defining criterion is that of distinctive elevation (i.e., the presence of either projecting or depressed morphology relative to the surrounding bone region). Such surface differentiation occurs at the tendon's footprint on the bone and its immediate surroundings, as described by histological studies and the "enthesis-organ" concept.[42] In contrast, relying on the coloration criterion alone is not advisable due to its dependence on taphonomic factors as well as its variable representation across the surface of fibrocartilaginous entheses.[10,26] It is worth noting that we have recently confirmed that our method can be precisely applied on 3D

BOX 1 The V.E.R.A. protocol for delineating and measuring 3D entheseal areas

Initially, the reconstructed 3D surface model should be imported into Meshlab (or another software package with advanced filtering and quantifying capacities) and the enthesis' location on the bone should be confirmed based on anatomical dissections and atlases. The presence of a visible surface differentiation must be confirmed in the same bone region (Figure 1a). Then, in case that color information is captured in the scanning procedure (but see above on limitations regarding this criterion), one may visualize color differentiation with the help of the "Equalize vertex color" filter (Figure 1b), followed by a close inspection of the entheseal area around 360°, from an oblique, close, and low angle of view (e.g., Figure 1c). Even though darker coloration does not usually characterize the entire span of the entheseal surface, its frequent occurrence is likely associated with the presence of calcified fibrocartilage at entheseal regions subjected to stress.[25] Subsequently, the observer should inspect differences in surface elevation (the most important criterion) and complexity (irregularity) using filters that color-map surface curvature patterns, such as "Discrete curvatures" or equivalent (Figure 1d). Another close, low, and oblique 360° inspection should follow in parallel for the demarcation of entheseal borders using the marker tool of Meshlab (Figure 1e). Importantly, after selecting the area with distinct elevation, the observer should additionally select a very thin zone of the surrounding flatter bone area across the entheseal perimeter (Figure 1f). Then, after cropping and isolating the selected region, the observer must color-map the resulting surface based on the principal direction of curvature (using the filter "Compute principal directions of curvatures" and then selecting "principal component analysis"), thus highlighting the graduation between the surrounding thin flatter zone (in dark blue; Figure 1g) and the elevated (or depressed) interior (in warmer colors). When the entire process is properly performed, the outerzone colored in dark blue captures the surrounding flat region along the entheseal borders (as in Figure 1g). Finally, this flatter zone (in dark blue) should be cropped (Figure 1h) and the final entheseal area must be superimposed onto the bone model, for final inspection (Figure 1i). It should be noted that there are cases in which the latter filter may not clearly distinguish the flatter zone around the enthesis (i.e., the surrounding dark blue zone is missing). Based on our observations, this occurs due to morphological particularities of the immediately surrounding bone regions (i.e., the unusual presence of elevations and/or depressions), which affect the filter's calculation of the principal directions of curvature in the selected bone region. This issue becomes immediately evident to the observer because the filter fails to produce a distinctive blue color along the margins of the delineated area. In such cases, one should resort to color-mapping based on the geodesic distance between the entheseal surface's limits and its midpoint, using the "Compute distance from borders" filter, and then cropping the flatter surrounding area (in this case, it appears in Meshlab as dark red). Examples of this filter's application can be found in figures of our previous research. [23] Finally, the resulting entheseal surface can be measured in square mm using the "Compute geometric measures" tool of Meshlab.

models developed using different scanning technologies (i.e. laser, structured-light, and computed-tomography scanning) as well as in cases of 3D surface models that lack color information (i.e., in computed tomography scans).[26]

After delimitation of their borders, the entheseal surface areas are cropped and computed in square mm. Box 1 and Figure 1 provide an original step-by-step description of our protocol for delimiting and computing entheseal 3D areas, aiming to further promote the accurate replication of V.E.R.A. in the future.

We have further extended this methodology through the use of landmark-based geometric morphometrics, to precisely analyze 3D form (size and shape) and allometry.[11] Specifically, the delineated entheseal surface areas are exported from Meshlab as PLY files and imported to the R-CRAN package Geomorph.[43] A set of fixed and geometrically corresponding landmark points are digitized along the entheseal outline, which act as the basis for calculating multiple surface-sliding semilandmarks. This approach showed substantial intra- and inter-observer repeatability (i.e., the error was below 5% across landmarks), allowing for a semi-automated, objective, and holistic representation of entheseal form (see text and figures of Section 4.3).[11]

3 | ENTHESEAL CORRELATIONS REFLECTING MUSCLE SYNERGIES (V.E.R.A. PROTOCOLS)

Previous research has suggested that intense long-term physical activity mainly influences the relative distribution of bone mineral density among an individual's different anatomical areas and less so the individual's overall levels of bone mineral density.[44–46] At the same time, considering the relatively low heterogeneity of bone density within each skeleton[45] and strong correlations across anatomical regions,[47,48] variation among skeletal sites of the same individual would hardly be expected to systematically exceed differences across distinct individuals of a sample under study. On this basis, directly comparing a single bone area across individuals may likely not be very informative on the effects of physical activity, as it is highly influenced by the multiple systemic factors regulating inter-individual variation in bone.[49] In contrast, the proportions among different bone areas of the same skeleton may be more indicative of the site-specific effects of physical activity.[22–24] This argument can be further clarified through the following hypothetical example involving entheseal 3D surfaces and bone size, which is found to be broadly correlated with

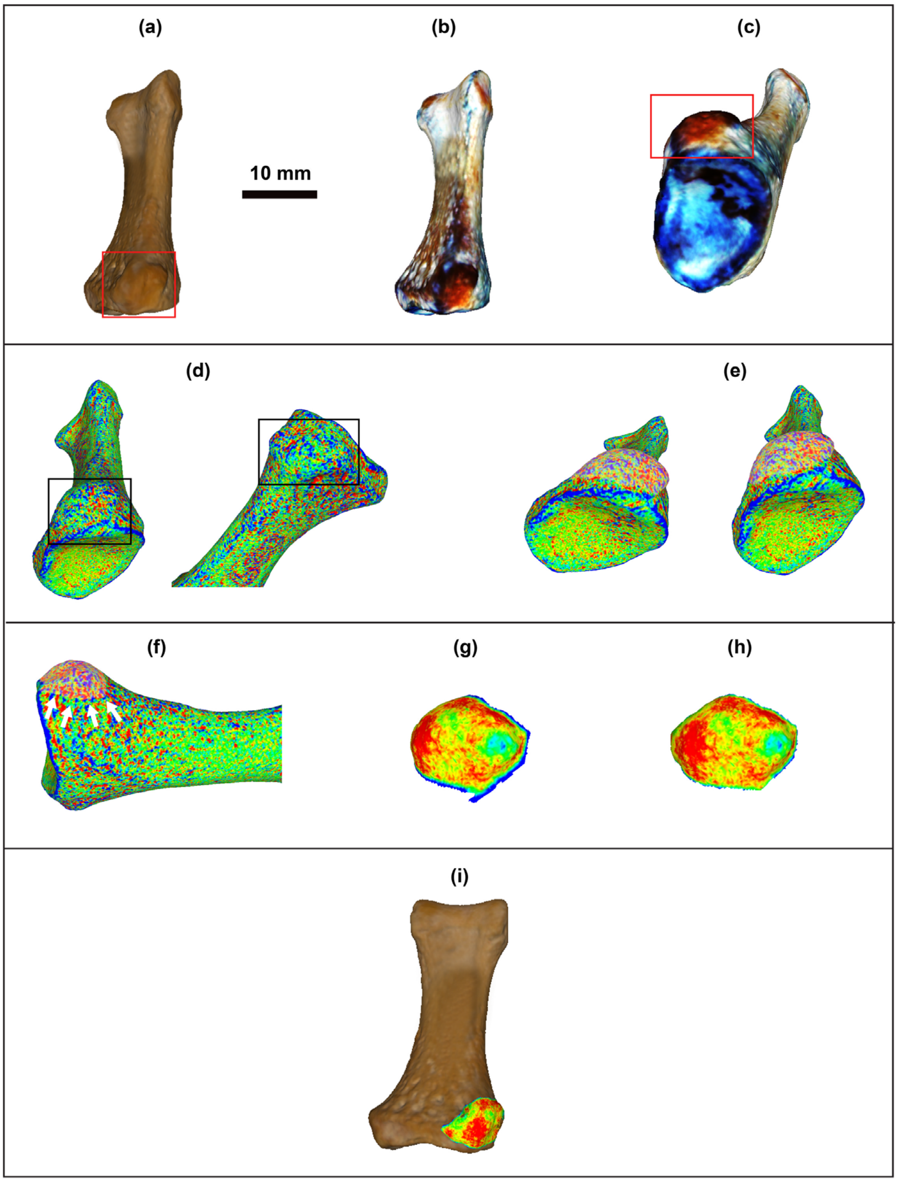

FIGURE 1 The exact steps of the delineation and quantification process of the 3D entheseal surfaces based on the criteria of elevation (i.e., the defining criterion), irregularity, and coloration[10,23] (for detailed text description, see section Box 1): (a) identification of the entheseal area on the bone (red box); (b) application of the "Equalize vertex color" filter for examining the coloration criterion; (c) 360° inspection for color deviation at the entheseal area (red box); (d) application of the "Discrete curvatures" filter (or equivalent) and 360-degrees inspection for examining elevation and irregularity deviations across the entheseal borders (black boxes); (e) selection of the area's border and interior over 360°, relying on the above two filters for tracking the presence of different surface elevation (i.e., accumulation of blue and green colors that tend to visually distinguish the structure from the surrounding flatter area) and coloration (i.e., darker shades); (f) additional selection of a very thin flatter zone (white arrows), around the enthesis (360°); (g) isolation of the selected region and color-mapping based on the principal curvature directions (filter "Compute principal curvature directions" and selecting "principal component analysis"; also see in Section 2 and Box 1 for alternative filtering options); (h) removal of the flatter zone surrounding entheseal borders (colored by the filter in dark blue; also see text for alternative filtering possibility); (i) superimposition with the bone model for final verification. The example used here is the enthesis of muscle *adductor pollicis brevis* on a left male pollical proximal hand phalanx

bone mineral density.[50–52] One could assume a comparison of two entheses ("A" and "B") between a hypothetical individual of overall smaller bone dimensions (Individual 1) and another of much larger overall bone volume (Individual 2). Assuming that the 3D surface size of both entheses in Individual 1 (e.g., A: 28 square mm; B: 44 square mm) would appear to be consistently smaller than in Individual 2 (e.g., A: 70 square mm; B: 74 square mm), a comparison of each enthesis separately would always show a greater entheseal size for Individual 2, irrespective of potential behavioral differences between the two individuals. However, calculating the relationship between entheses within each individual skeleton (e.g., the ratio between entheses A and B) would reveal that enthesis B is proportionally much larger in Individual 1. That difference in the relative distribution of bone surface between the two entheses would be independent from each individual's overall skeletal dimensions. In our experimental studies on laboratory rats and turkeys,[23,24] such proportional entheseal differences were identified between activity specimens and controls (see also below in Section 4.2 and Figure 2a). It is worth noting that such differences also occurred among specimens of very similar body sizes and/or bone lengths,[23,24] indicating that a simple size-adjustment of entheseal values (e.g., see Figure 2b) would not sufficiently track the functionally meaningful patterns revealed by the multivariate analyses of correlations among different entheses.[23,24] This is likely due to the effects of other factors affecting inter-individual entheseal variation (in addition to body size), such as genetic background, sex, age, nutrition and hormones.[3]

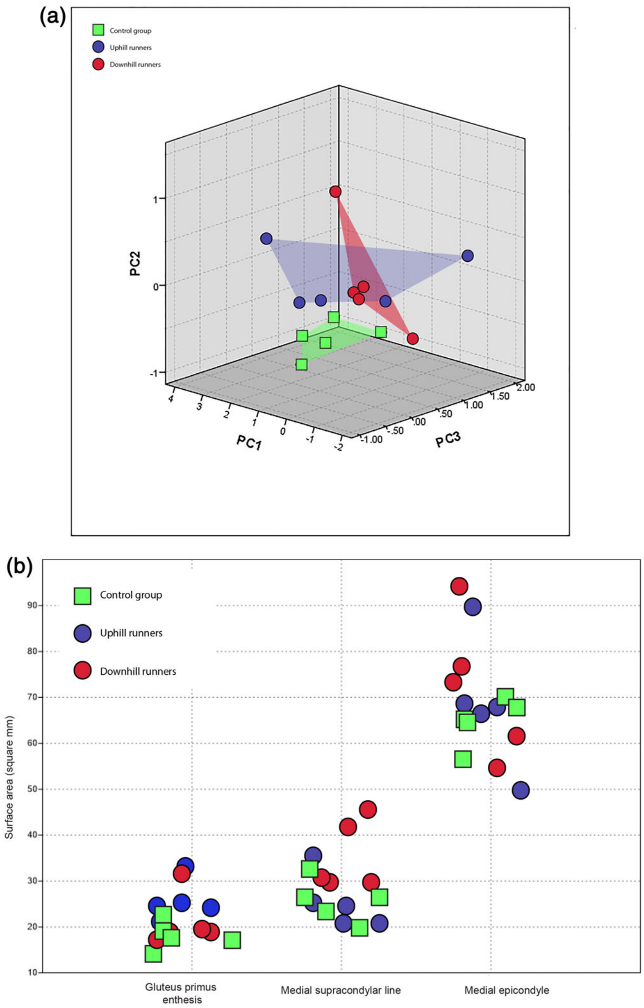

FIGURE 2 The results of past experimental research on laboratory turkeys (figures previously published in Karakostis et al.[24]), demonstrating that a multivariate 3D analysis (i.e., PCA without prior group assumptions) involving three entheses of closely synergistic muscles (a) can clearly distinguish between exercised specimens (runners) and controls. In contrast, comparing activity groups within each entheseal structure separately (b) could not possibly identify a distinctive functional signal. The two panels of this figure correspond to two of the previous study's figures.[24] In the jitter plot of the lower panel (b), the raw values of each specimen (vertical axis) is presented for each enthesis (horizontal axis)

Importantly, the fact that habitual activity mostly reflects on the distribution of bone mineral across different skeletal parts[44–46] encourages the investigation of relationships among entheses corresponding to muscle synergy groups. Essentially, one would expect that systematic coordination of a muscle group for a certain type of activities (e.g., precision-grasping tasks) would lead to the emergence of a correlation among the corresponding muscle entheses.[22] Similarly, another individual systematically relying on different muscle synergies (e.g., intense power-grasping labor) would be expected to show comparatively different entheseal correlations.

In spite of the above arguments as well as the extensive use of multivariate analyses in most fields and lines of biological research (e.g., taxonomy,[53,54] genetics,[55] cross-sectional biomechanics,[56,57] or forensic anthropology[58,59]), such analyses are extremely rare in entheseal studies. In fact, the vast majority of past anthropological literature on entheses—including 3D studies on humans or laboratory animals—relied exclusively on either univariate or bivariate comparisons of single entheseal structures across individuals.[12,17–19] One exception involves a past study that performed a multivariate analysis of entheseal robusticity scores using individuals documented for their age and occupation-at-death.[60] These authors transformed the observed robusticity scores into binary values, which were then subjected to subsequent multivariate procedures. Their comparisons revealed certain significant differences between farmers and individuals with physically undemanding occupations. However, the statistical design of this previous approach did not indicate the exact combinations of entheses correlating differently between the groups and their potential functional relevance to behavioral variation.

In this framework, V.E.R.A. combines the above-described protocol for precise 3D quantification of entheses with multivariate statistical methods that do not assume groups a priori (i.e., principal component analysis, PCA, on a correlation matrix).[10,22,24,26] Additionally, discriminant function analysis (DFA) is also occasionally performed as a supplement to the PCA, when applicable.[26] These statistical analyses (PCA and DFA) are carried out both before and after size-adjustment (based on the geometric mean).[22] Our first application of this 3D multivariate approach on a medieval sample from Spain,[10] revealed two distinct morphometric trends among entheses, directly corresponding to two distinct muscle synergy groups commonly related to power versus precision grasping movements (i.e., functionally meaningful group correlations among entheses). On an experimental level, our previous research demonstrated that multivariate 3D entheseal analyses (PCA) can distinguish a clear functional signal in entheses (Figure 2a), which is not observable via any type of univariate or bivariate statistical comparisons of single entheseal structures across different specimens or groups (Figure 2b). Such bivariate statistical approaches may involve probability tests of correlation (e.g., Pearson's correlation coefficient, Spearman's rank correlation coefficient, and others), group comparison (student *t*-tests or Mann–Whitney *U* tests), or various linear models that do not involve the simultaneous analyses of different entheses for each individual.

4 | VALIDATION

4.1 | Validation on skeletons with documented life-histories

In order to explore V.E.R.A.'s potential for reconstructing habitual activity patterns, we applied it to a uniquely documented bioarchaeological sample from mid-19th century Basel (hereafter the Basel collection), Switzerland, where not only sex, age and occupation at death are known, but also family relationships, socioeconomic background, and, most importantly, lifetime occupation.[22,61] Unlike previous work relying on occupation-at-death records,[62,63] we had access to archived documentation that included duration of different occupations, position at work, and hiring company or institution. To our knowledge, this level of lifelong documentation is unique in biological anthropology and enables a deeper understanding of each reference individual's lifestyle and occupational behavior.

When applying V.E.R.A. to a sample of 45 male individuals of relatively young adult age (i.e., between 18 and 48 years old), we found a close statistical association between size-adjusted entheseal multivariate patterns and the nature of their lifelong occupational activity, without relying on prior group assumptions. Individuals involved in heavy manual labor (e.g., lifelong construction workers) presented a multivariate entheseal pattern related to muscles coordinated for power-grasping. In contrast, long-term precision workers showed a pattern of entheses closely conforming with precision grasping using the thumb and the index finger (Figure 3). This functional signal was only observable via multivariate analysis (PCA) and not when comparing each single enthesis across specimens. Importantly, these size-adjusted multivariate patterns were not associated with population, biological age, genetic relatedness among individuals (based on detailed genealogical records), body height, body mass, hand bone length, pathology (based on the individuals' official medical records), or socioeconomic differences.[22]

4.2 | Experimental validation

Experimental support is a fundamental step for assuring the reliability of methods attempting to reconstruct something as variable and complex as human behavior from skeletal remains.[2] Several past experimental models on animals, including sheep, mice and turkeys, did not find evidence for a correlation between activity and entheseal expression.[17–19] The lack of statistically significant differences between exercised and control groups was interpreted as evidence against a functional character of muscle attachments. However, these previous experimental studies focused on bivariate comparisons of single entheseal structures and not on multivariate patterns reflecting

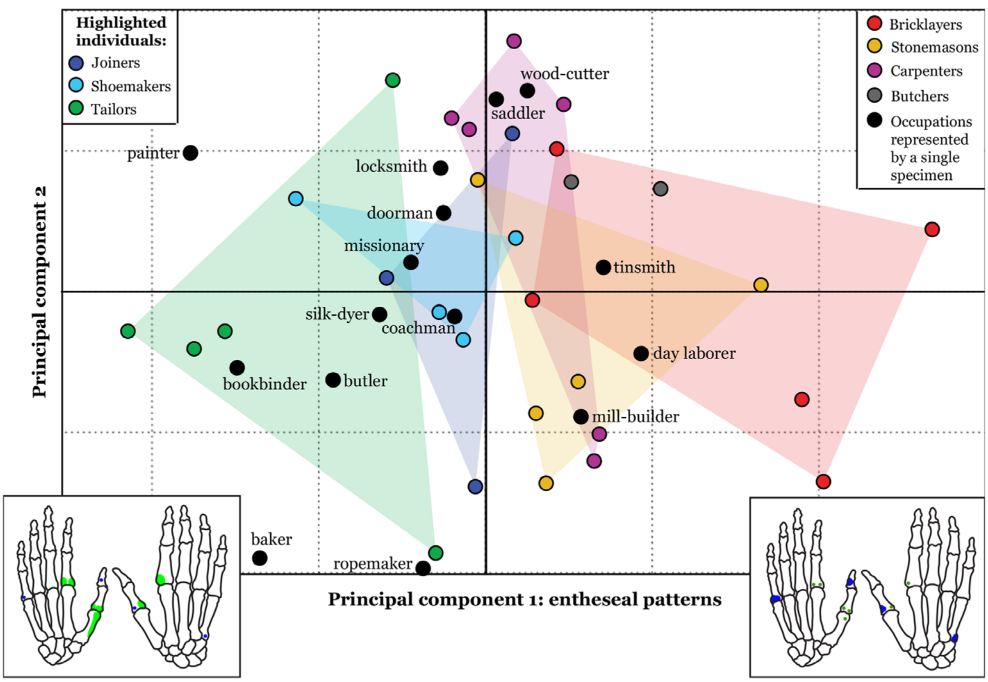

FIGURE 3 The results of previous research on skeletons with a uniquely detailed level of lifelong occupational documentation, involving different occupations, their durations, position at work, and hiring organization or institution (figure previously published in Karakostis et al.[22]). No prior group assumptions were made, and individuals were colored by lifetime occupation. Before analysis, values were adjusted for size using the geometric mean. The side illustrations summarize the entheseal patterns represented by the two directions of PC1, with green color representing a precision-grasping muscle group and blue color referring to a power-grasping muscle group.[22] Almost all individuals with positive scores of principal component 1 were long-term heavy construction workers and presented a dominant power-grasping entheseal pattern (in the bottom right illustration, entheses in blue are proportionally larger), whereas individuals with negative values were involved in precise and/or semi-mechanized occupational tasks (in the bottom left illustration, entheses in green are proportionally larger)[22]

muscle synergies. The complete absence of a functional signal in muscle attachment bone areas suggested by these studies contradicts an entire body of cross-disciplinary literature reporting substantial bone loss (at the millimeter scale) in the entheses of adult mice after artificial muscle paralysis[64]; significant correlations between entheseal variables and bone cross-section geometry that is widely associated with stress,[23,65–68] considerable biomechanical forces acting on entheseal bone surface during muscle contraction (inferred from finite element analyses)[69]; and concrete histological and/or experimental evidence of reaction to biomechanical stress and bone remodeling on entheseal bone surfaces.[25,30,70]

We therefore aimed to further test this hypothesis, and at the same time experimentally validate our 3D multivariate approach for analyzing entheses. We relied on existing experimental data developed under controlled laboratory conditions in previous research using rats and turkeys.[19,71] The results of both applications[23,24] identified a clear functional signal in entheseal multivariate patterns, which was not identifiable when comparing individuals for each entheseal structure separately. Specifically, our first study relied on muscle stimulation of certain lower limb muscles from one anatomical side of a Wistar rat sample. Our multivariate analysis distinguished between stimulated and unstimulated limbs under blind study conditions involving different institutions (Figure 4), while revealing strong statistical correlations among the observed entheseal patterns, muscle volume, and cortical thickness in bone regions subjected to statistically significant biomechanical stress during the experiment (Figure 5 and its legend).[23] In our second experimental research, we focused on reconstructing physical activity in a sample of turkeys participating in

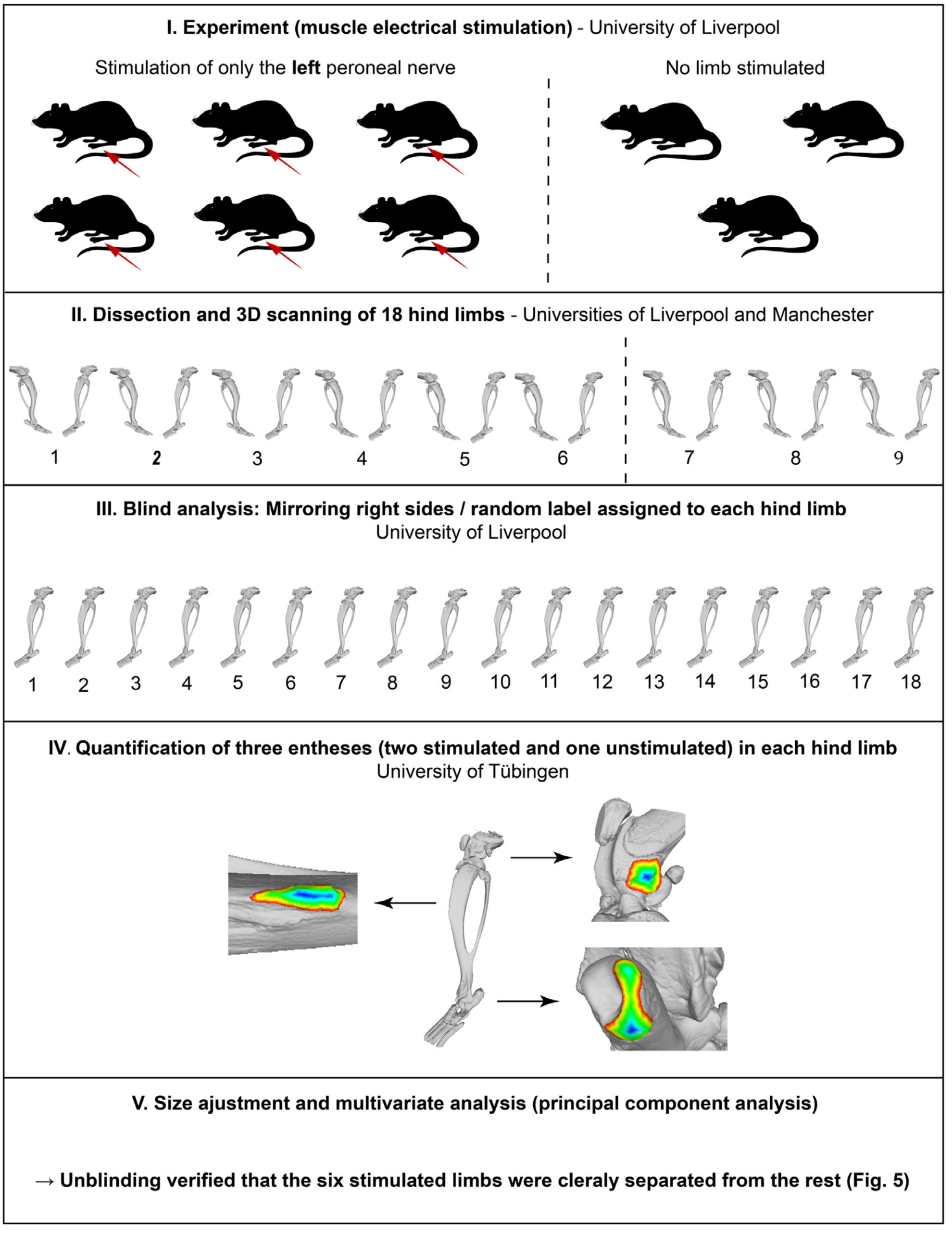

FIGURE 4 The methodological steps of the past experimental research involving stimulation of certain muscles in laboratory rat specimens (figure previously published in Karakostis et al.[23]). The employed analytical strategy relied on double-blind analytical conditions involving two distinct research groups and institutions. The results can be found in Figure 5

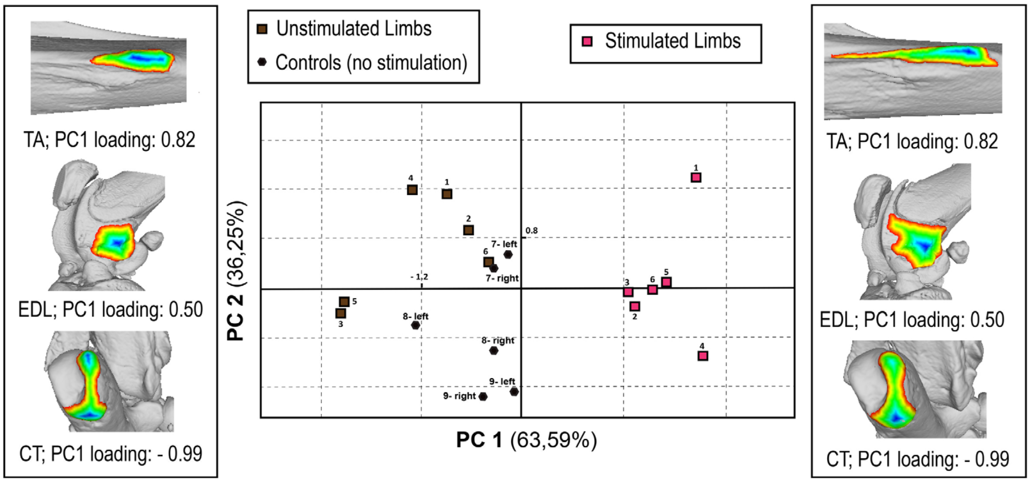

FIGURE 5 The results of experimental research on rats (figure previously published in Karakostis et al.[23]), relying on three entheses. No prior group assumptions were made and, in agreement with a double-blind analytical protocol (Figure 4), the identity of each specimen cases was revealed only after entheses were measured and analyzed using PCA. The side figures represent the entheseal patterns represented by each direction of principal component 1 (PC1). Stimulated specimens exhibited a multivariate pattern with proportionally larger entheses of the two muscles stimulated in the experiment.[23] Importantly, the values of PC1 were found to strongly correlate with muscle volume (*p*-value <.01; *r*-value = –0.61) as well as cross-sectional cortical thickness in a bone region subjected to significant biomechanical stress during the stimulation experiment (*p*-value <.01; *r*-value = 0.76)[23,71]

uphill- and downhill-running activities, or serving as controls.[24] The same sample had previously been used by a study reporting no functional signal in entheses without using multivariate statistics.[19] In contrast, when this sample was re-assessed using our multivariate approach, clear femoral entheseal differences between controls and runners were revealed (Figure 2a).[24]

4.3 | Entheseal form and biomechanical efficiency

Past research has sought to evaluate the biomechanical significance of entheseal morphology. Some of these previous studies investigated the association between 3D entheseal measurements and muscle variables related to biomechanical efficiency.[12,17,72] These muscle measurements typically involve physiological cross-section area (PCSA) and fiber length. The former is thought to be indicative of the maximum force that a muscle can generate, while the latter is also correlated with the muscle's maximum excursion (associated with contraction velocity).[73] Deymier-Black et al.[72] analyzed a sample comprising several different non-primate mammal species and reported a strong correlation between PCSA values and entheseal 3D areas, proposing that entheseal variation is highly indicative of interspecies differences in the level of muscle forces generated.[64,70,72] In contrast, Williams-Hatala et al.[12] focused on human cadaveric individuals and found no linear bivariate correlation between hand entheseal measurements and muscle architecture (PCSA and fiber length). They therefore concluded that the forces generated by a muscle are not directly associated with its attachment's morphology.

Nevertheless, the latter study[12] exclusively relied on individuals of very advanced biological age, despite the extensive known consequences of advanced age on soft tissue, bone, and physical activity.[70,74,75] Fundamentally, none of the above works considered the muscle's moment arm length, one of the two parameters needed to calculate torque (along with muscle force), a foundation of biomechanical efficiency.[73,76] As it was statistically demonstrated in our recent biomechanical studies,[77,78] joint moment arm naturally varies with the level of entheseal surface projection[11,77–80] and is biomechanically shown to substantially affect torque calculations even between identical systems exerting the same exact muscle forces.[76] Therefore, biomechanical assessments neglecting the moment arm's influence on muscle force-producing capacities essentially silence the very contribution of entheses to biomechanical efficiency.

Another approach for investigating the relationship between entheseal projecting morphology and cumulative biomechanical stress involves the histological microscopic analysis of entheses. A plethora of histological studies has focused on human muscle attachments from different anatomical regions,[1,27,30,35,42,64,66,69,72] helping to shed light into fundamental aspects of human entheseal structure, function, pathology, and etiology. Multiple studies have concluded that the concentration and distribution of fibrocartilage in fibrocartilaginous entheses is correlated with the levels of biomechanical stress inflicted on entheses during long-term muscle contraction.[25,27,35,69] The zone of uncalcified fibrocartilage serves to dissipate the biomechanical forces inflicted by the tendon's bending collagen fibers,[27] while calcified fibrocartilage (CF) functions as the point of union between bone and soft tissue, anchoring the tendon onto the bone.[1]

Even though CF is occasionally preserved in dried bone tissue,[1] its value in anthropological analyses of human bone remains is underrepresented in the literature.[25] Until recently, researchers had overlooked the intraspecific inter-individual variability in the distribution of CF, which may provide insight into stress levels at a particular muscle attachment during life. A pilot study by Karakostis et al.[25] on a small cadaveric sample of non-pathological hand entheses explored the relationship of CF and entheseal morphology, in order to further test the hypothesis that the proportion of bone elevation at muscle attachments is correlated with biomechanical stress. We found that the elevated portion of hand entheses presents greater evidence of stress, indicated by greater levels of CF, while individual entheses with greater proportional projection presented substantially higher levels of CF (both in raw and relative values; see Figure 6). These findings are in agreement with and broadly confirm the biomechanical principle that greater entheseal projection naturally results in larger muscle moment arm[77–79] and, thus, higher force-generating capacities.[76] Future histological research on increased sample sizes and on other human entheses can further validate these observations, potentially providing new statistical means (e.g., regression equations) for predicting cumulative biomechanical stress based on the projecting morphology of entheses and their associated moment arm. The use of such statistical tools in the fossil record may allow more reliable functional interpretations of variation in bone robusticity.

Until present, however, the degree of muscle attachment elevation in fossil hominins has been measured solely based on bone linear dimensions.[79] Even though such approaches have previously provided fundamental insights,[68,79] new and precise tools for recording 3D shape can provide more accurate and detailed representations of entheseal proportional projection. For this purpose, we previously explored shape variation in hand entheseal surface areas using landmark-based 3D geometric morphometrics and focusing on three entheses in the first and the fifth hand ray (following the process outlined at the end of Section 2).[11] Applying this novel technique on a recent modern human sample, we were able to calculate both 3D size and shape variables, while the exact shape differences across

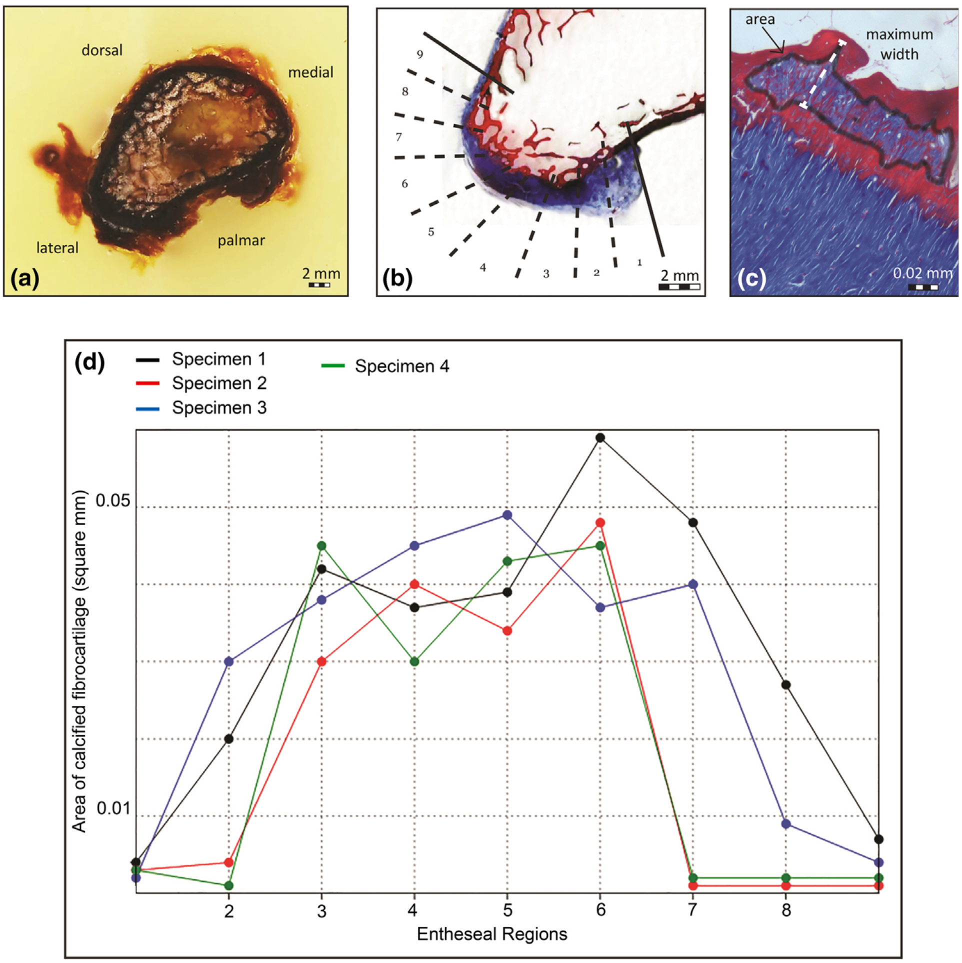

FIGURE 6 Some of the results of previous histological microscopic research on human cadaveric hand entheses.[25] The upper three panels (a–c) were previously published in Karakostis et al.[25] and demonstrate: (a) a paraffin-embedded tissue block of the thumb's proximal phalanx including the attachment site for muscles *abductor-pollicis brevis* and *flexor pollicis brevis*; (b) staining of a histological section and virtual separation of the enthesis into nine equal regions; and (c) digital measurements of calcified fibrocartilage, which is a widely-proposed direct proxy of cumulative biomechanical stress[25,27,30,69]). The bottom panel (d) is extracted from the fifth figure of Karakostis et al.[25] and demonstrates the regions within each enthesis showing greater levels of calcified fibrocartilage. Based on that study's visual macroscopic and microscopic observations, all entheseal regions with high concentration of calcified fibrocartilage also exhibited high bone elevation. Furthermore, across individuals, the entheses containing a greater number of elevated regions also presented higher total concentrations of calcified fibrocartilage

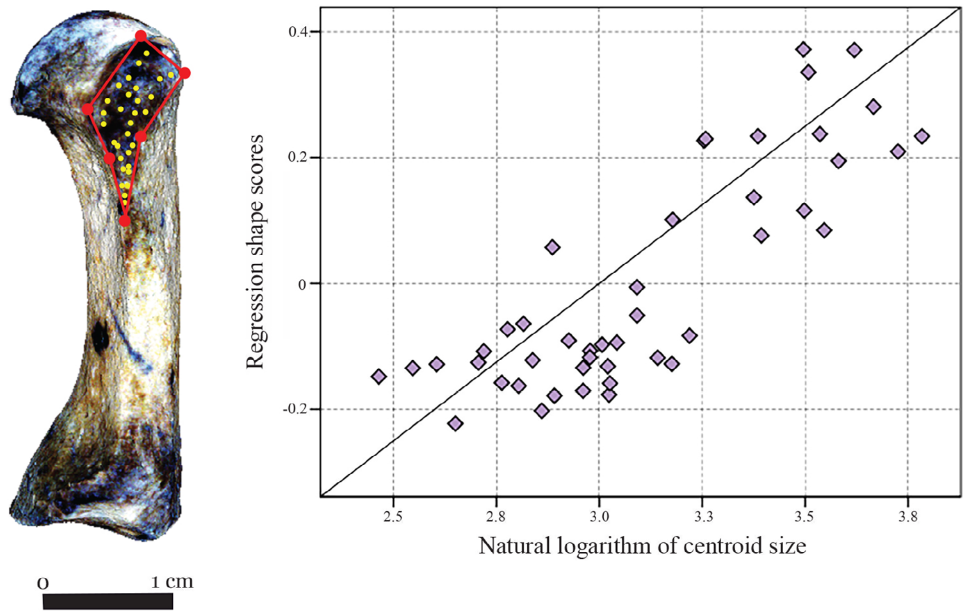

FIGURE 7 Results of a recent 3D geometric morphometric analysis of the *opponens pollicis*' insertion site on the first metacarpal.[11] The two depicted panels (left and right) were extracted from figures previously published in Karakostis et al.[11] Left: Lateral view of a human right first metacarpal (distal is up), indicating the locations of the selected fixed landmark points (red dots) and surface-sliding semi-landmarks (yellow dots). For clearer visualization, the imaging filter "Equalize vertex color" has been applied on the bone model's surface (using the software Meshlab; see Figure 1). Right: Bivariate plot showing the results of an allometric regression analysis, revealing a statistically significant association between the 3D size of *opponens pollicis*' enthesis (i.e., the "natural logarithm of centroid size"[43,81]) and its shape variable most strongly associated with size (i.e., "regression shape scores"[43,81]). Based on visualizations of shape change, the latter variable was shown to mainly reflect the attachment area's degree of proportional bone projection[11]. The extent of bone projection is shown to be strongly correlated with longer moment arms and higher force-producing capacity for the attaching muscle[77,78]

individuals could be intuitively visualized in multiple parts of an enthesis at the same time.[43] We[11] found a statistically significant interaction between 3D size and shape in human hand entheses (i.e., allometry), demonstrating that larger entheses are consistently steeper (more projecting) than their smaller counterparts. Based on our more recent biomechanical studies, higher entheseal projection is strongly correlated with greater joint moment arms and force-generating capacities for the attaching muscles.[77,78] For the insertion of the muscle *opponens pollicis*, which plays a central role for thumb opposition,[40] the association between entheseal size and proportional projection explained approximately 26% of its morphological variability (Figure 7). The future application of this geometric morphometric approach on fossil hominins could provide new insights into functional differences across species or populations, helping to reevaluate hominin biomechanical adaptations.

5 | LIMITATIONS AND PATHWAYS FOR IMPROVEMENT

In spite of the promising findings of the above interdisciplinary studies, several important aspects of 3D entheseal variation remain unexplored and should be further addressed by future research. Essentially, the highly documented sample used in our comparative studies[22,26] exclusively relied on male individuals of central European ancestry that were below 50 years of age. Therefore, an even deeper understanding of entheseal variability could emerge through future research focusing on the effects of sexual dimorphism, interpopulation variability, and biological age on the multivariate associations among different 3D entheseal surfaces. Furthermore, even though V.E.R.A. is readily applicable to any other human or animal entheseal structure (as shown by our studies on lower limb bone entheses of rats and turkeys[23,24]), it has currently mainly been applied to the muscle attachments of the human hand. Therefore, future research should expand the investigation of entheseal correlations across the human skeleton, achieving a more holistic reconstruction of individual behavior based on skeletal remains.

Furthermore, despite the strong functional signal identified in our two experimental applications,[23,24] we believe that future experimental research is needed to further elucidate the observed interaction among entheses, muscle architecture, and/or habitual physical activity. For instance, future experiments can rely on sessions with longer durations that involve different species and a variety of activity regimes. Such analyses are needed to further explore V.E.R.A.'s limits in distinguishing between different activity groups and identifying functional similarities among species, providing an important theoretical basis for interpreting interspecies differences in the fossil record.

It should also be highlighted that the initial study introducing our 3D multi-entheseal approach[10] involved a higher number of hand entheses (i.e., 17) compared to our more recent studies that relied on either nine or three muscle attachment sites.[22,24,26] This decision was primarily based on the central functional importance of the muscles associated with these nine attachment sites (e.g., thenar or hypothenar muscles), as well as on our results demonstrating that these entheses effectively contributed to patterns associated with muscle synergy groups that reflect standard human power or precision grasping movements.[10,22,26] Furthermore, in our previous study on Neanderthal and early modern human hand entheses[26], one of the analyses relied on a reduced set of three insertion sites so as to maximize the representation of fossil hominins in our samples. Nevertheless, future studies would greatly benefit from incorporating more hand entheses in their biomechanical hypotheses and analyses, potentially allowing for an even greater resolution of habitual grasping variability.

Finally, it must be emphasized that a reliable application of the methods discussed here requires a brief period of training in virtually delineating 3D entheseal areas using the steps of the protocol outlined in Box 1 and Figure 1. Based on our research experience involving multiple collaborators from different scientific disciplines, this training session would ideally be provided by V.E.R.A.'s developers or experienced users. Therefore, we would strongly encourage researchers interested in applying our methods for analyzing entheses to directly contact the authors of this article. Nevertheless, we also believe that students and researchers with an adequate background in 3D imaging and morphometric techniques would most likely have the capacity to also self-learn this new approach, after first familiarizing with the use of 3D imaging filters and virtual measuring tools. To further facilitate this learning process and encourage researchers to consider this proposed avenue for reconstructing behavior in the past, we have detailed V.E.R.A.'s delimiting protocol in Box 1 and Figure 1. We strongly believe that the minimum quality criterion for any new user would be significant intra- as well as inter-observer 3D measuring repeatability. Fundamentally, after familiarizing with the process for several entheses, our research experience has shown that it is possible to accurately implement the protocol on muscle attachment sites of different species and anatomical regions.[23,24] We would be more than willing to assist future researchers in conducting precision tests using different human and/or animal attachment sites.

6 | NEW HORIZONS FOR HUMAN EVOLUTIONARY RESEARCH

The potential value of our new approach for human evolutionary studies was demonstrated by the recent application of V.E.R.A. on a set of Neanderthal and early modern human hand remains from diverse geographic and chronological backgrounds.[26] Our multivariate 3D analyses of entheseal surface measurements showed that all Neanderthals exclusively overlapped with recent long-term precision workers, presenting a distinctive precision-grasping pattern involving the thumb and the index finger muscle entheses. This result reconciled Neanderthal manual anatomy, previously interpreted as indicative of habitual power grips,[82] with archaeological evidence indicating that Neanderthals were capable of sophisticated cultural behavior requiring habitual precise manipulation.[83] It is also in agreement with recent experimental archaeology studies[84] showing that the production and use of flakes such as those used by Neanderthals mainly requires forceful precision grasping involving the thumb and the index finger. Furthermore, we showed that some early modern humans presented power-grasping entheseal patterns and others showed precision-grasping tendencies, likely reflecting the frequently proposed emergence of greater inter-individual occupational variability in Upper Paleolithic populations.[26,77]

Overall, we strongly believe that V.E.R.A., our multivariate, quantitative method of analysis of entheseal variation (Box 1 and Section 3), validated both on the basis of a uniquely documented historical sample[22] as well as experimental evidence,[23,24] opens entirely new possibilities for human evolutionary and bioarchaeological research. Even though it has as yet only been applied to the human hand, V.E.R.A. can be extended to evaluate other parts of the skeleton (as also demonstrated in our experimental studies focusing on animal lower limb bones[23,24]), addressing questions about the evolution of behaviors in the fossil record or the presence of specialized activities in past human societies. For instance, our entheseal methodology could provide a new basis for exploring patterns of obligatory bipedal locomotion in early hominins,[85,86] the production and use of distinct stone tool industries in the fossil record,[40,87] hominin mobility and subsistence strategies,[86,88] behavioral differences between modern humans and other hominin species,[26,89–91] as well as the emergence of proposed aspects of behavioral modernity associated with division of labor, occupational variability and the use of sophisticated artifacts.[91,92] Therefore, we believe that V.E.R.A. provides a new and reliable avenue for the interpretation of muscle attachment 3D morphology that can lead to novel functional information linking human skeletal remains with their associated environment and cultural contexts.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ORCID

Fotios Alexandros Karakostis https://orcid.org/0000-0003-3913-4332

Katerina Harvati https://orcid.org/0000-0001-5998-4794

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AUTHOR BIOGRAPHIES

Fotios Alexandros Karakostis is Junior Research Group Leader in the DFG Centre for Advanced Studies "Words, Bones, Genes, Tools: Tracking Linguistic, Cultural and Biological Trajectories of the Human Past," at the Eberhard Karls University of Tübingen. His work focuses on biomechanics, functional morphology, forensics, and the evolution of the hand.

Katerina Harvati is Professor of Paleoanthropology at the Senckenberg Centre for Human Evolution and Palaeoenvironments, Eberhard Karls University of Tübingen. She is Director of the Institute for Archaeological Sciences, as well as co-Director of the DFG Centre for Advanced Studies "Words, Bones, Genes, Tools: Tracking Linguistic, Cultural and Biological Trajectories of the Human Past" at the same University. Her research focuses on human skeletal variation and its relationship to population history, adaptation, and behavior; on Pleistocene human evolution, Neanderthal paleobiology, and modern human origins; and on the paleoanthropology of South-East Europe.

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