DNA-predicted hair and eye colour. Genetic data can also be used to infer phenotypic traits such as hair and eye colour[31,32]. There are no contemporary portraits of Richard III (ref. 33), all of them post-dating his death by some 25 years or more (see Supplementary Note 4 and Supplementary Fig. 6). Dendrochronological analysis has confirmed that the earliest of all known portraits of Richard III to have survived are the Society of Antiquaries of London (SAL) Arched-Frame portrait and the portrait in the Royal Collection, both thought to date within a few years of each other in the 1510s. The SAL portrait is very different from other paintings of the king, which appear to derive from an original type represented by the portrait held by the Royal Collection. The SAL portrait also has not been subject to significant later overpainting[33].

Eye and hair colour DNA typing was carried out using probes designed for the HIrisPlex[31] SNPs and, where necessary, followed by directed PCR using newly designed primers to generate amplicons under 100 bp in length, followed by sequencing on an Ion Torrent PGM (see Supplementary Table 9). Phenotype predictions were produced from the IrisPlex and HIrisplex[31] statistical models[34]. These results show that Skeleton 1 had a 96% probability of having blue eyes together with a 77% probability of having blond hair (see Fig. 2a–b and Supplementary Note 4). Figure 2a–d shows blue eyes of contemporary Europeans whose DNA predictions fall within the range of high blue probability estimated from the Skeleton 1 profile. Similarly, Fig. 2e and f shows blond hair colour within the range of the high blond probability estimated from the Skeleton 1 profile. However, current hair colour DNA predictions resemble childhood hair colour and it is important to note that in certain blond individuals, hair colour can darken during adolescence. It is therefore possible that Skeleton 1 had brown hair as represented in Fig. 2g and h as seen in contemporary Europeans with a similarly high blond probability as obtained for Skeleton 1. The painting of Richard III that most closely matches the genetically predicted eye and hair colour results is the SAL Arch-Framed portrait (see Fig. 2i and Supplementary Note 5).

Statistical analysis. To obtain a probability that Skeleton 1 is that of Richard III, we considered the non-genetic data (radiocarbon data[6], estimated age at death, sex, presence of scoliosis[8] and presence of perimortem wounds[7]) together with the genetic data (mtDNA and Y-chromosome). For each data type, we computed likelihoods for the observed data under hypothesis 1 (H1—that Skeleton 1 is Richard III) and under hypothesis 2 (H2—that Skeleton 1 is not Richard III). Although the mtDNA evidence favours H1, the Y-chromosome evidence provided limited evidence against H1, and our conservative analysis of the genetic evidence only gave moderate support for H1 (likelihood ratio, LR = 79; see Supplementary Methods). Using a sceptical prior probability of 0.025 that skeleton 1 is that of Richard III, we obtained a posterior probability of 2/3 that H1 is true. On the other hand using a prior probability of 0.5, the genetic evidence lead to almost 99% probability that H1 is true. This analysis is highly conservative because, first, it used a low rate for false-paternity events, and second, the probability of a mtDNA match by chance (match probability) used was greater than 0.001, much higher than would be suggested by the absence of control region matches in the European database ($n = 26{,}127$, LR = 6,847). Furthermore, this analysis does not take into account the whole-genome mtDNA match with one modern relative and a single-base difference with a second. We note that if we ignore the Y-chromosome evidence, because of its susceptibility to false-paternity events, the contribution of the genetic data strengthens considerably (LR = 478). The non-genetic evidence strongly supports H1 (LR = 85,000). All the evidence combined is therefore extremely strong in supporting H1 (LR = 6.7 million). This LR leads to a probability that H1 is true between 0.999994 (sceptical prior) and 0.9999999 (0.5 prior, see Supplementary Table 10). All likelihoods were computed under conservative assumptions (discussed in the Supplementary Methods) and therefore, these reported values are almost certainly lower than justified by the evidence.

Discussion

The search for the remains of Richard III can be likened to a missing person's case, with such investigations becoming more difficult the longer the time between the investigation and the time of death of the individual[35,36]. Given the 527 years that had elapsed since Richard's death at Bosworth, this case is of special interest in that it is the oldest DNA identification case of a known individual to date. As with any such case, all quantitative strands of evidence should be drawn on to reach a conclusion regarding the identity of any putative candidate. This report is the first that draws all such available strands together and estimates the statistical support for the skeletal remains discovered in 2012 being those of the last Plantagenet king, Richard III. d1

In drawing the evidence together, historical documents indicated that we would be looking for the remains of an individual who was described, during his lifetime, as having one shoulder higher than the other, who, in 1485, aged 32, died in the heat of battle before being brought back to Leicester to be buried in the choir of the church of the Grey Friars. In September 2012, the remains of an individual fitting all these criteria were found. Subsequently, in addition to the compelling archaeological evidence, laboratory analyses provided information on radiocarbon dating[6], isotopic analyses[9], the degree and nature of the scoliosis[8] as well as the injuries sustained[7]. We present the genetic analysis of the remains and the only known female-line relatives of Richard III and find a positive mtDNA match. Whilst there was no Y-chromosome match between the skeletal remains and five genealogically determined male-line relatives, given the known possibility of a false-paternity over several generations, this did not prove to be a highly significant factor. d2 d3 One can speculate that a false-paternity event (or events) at some point(s) in this genealogy could be of key historical significance, particularly if it occurred in the five generations between John of Gaunt (1340–1399) and Richard III (see Supplementary Fig. 2). A false-paternity between Edward III (1312–1377) and John would mean that John's son, Henry IV (1367–1413), and Henry's direct descendants (Henry V and Henry VI) would have had no legitimate claim to the crown. This would also hold true, indirectly, for the entire Tudor dynasty (Henry VII, Henry VIII, Edward VI, Mary I and Elizabeth I) since their claim to the crown also rested, in part, on their descent from John of Gaunt. The claim of the Tudor dynasty would also be brought into question if the false paternity occurred between John of Gaunt and his son, John Beaufort, Earl of Somerset. If the false paternity occurred in either of the three generations between Edward III and Richard, Duke of York, the father of Edward IV and Richard III, then neither of their claims to the crown would have been legitimate. d5

Analysing all the available evidence in a Bayesian framework, even using highly conservative measures, we conclude that the evidence is overwhelming that Skeleton 1 from the Grey Friars site in Leicester is that of Richard III, thereby closing a 500-year-plus missing person case. d4 d6

Methods

Laboratory locations. All DNA work involving the modern relatives was carried out at the University of Leicester. Male-line relatives were typed using Promega PowerPlex Y23 and for SNPs defining the main European Y-haplogroups in

Figure 6. Example face array from the spontaneous recognition task in Experiment 2. (**a**, **c–f**) Corneal reflection images showing bystanders AS, CK, AC, MA, and PD from Experiment 1. (**b**) Corneal reflection image showing author RJ.
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Results

To ensure that the familiarity manipulation was not compromised by items from the opposite category, any faces that were unknown to an observer in the *Familiar* group (<11%) or known to an observer in the *Unfamiliar* group (<2%) were excluded from analysis. Accuracy in the *Unfamiliar* condition was well above chance level of 50%, despite the demanding nature of the matching task [$n = 16$; mean = 71%; s.d. = 11.1; two-tailed $t(15) = 7.64$, $p < .0001$, $d = 1.91$]. Accuracy in the *Familiar* condition exceeded both chance performance [$n = 16$; mean = 84%, s.d. = 2.1, two-tailed $t(15) = 11.15$, $p < .0001$, $d = 2.79$] and performance in the *Unfamiliar* condition [two-tailed $t(30) = 3.02$, $P < .01$, $d = 1.10$], confirming that bystanders' faces could be reliably distinguished from similar foils.

Experiment 2: Spontaneous recognition

Previous studies have shown that face matching accuracy is a reliable proxy for face recognition accuracy [11–13]. Here, we had the opportunity to test recognition directly, by presenting eye reflection images in a face naming task. This experiment was motivated by an anonymous reviewer who reported recognizing author RJ from Figure 2b. To test spontaneous recognition more formally, we presented eye reflection images of RJ and 5 other males in a lineup-style array. Observers who were *familiar* with the face of RJ, and *unfamiliar* with the other faces, were asked to name anyone in the array whom they could identify. We expected that if eye reflection images can be spontaneously recognized, then i) the hit rate (correct naming of RJ) should be high, and ii) the false positive rate (mistaken identification of unknown faces) should be low.

Method

Design and materials. Corneal reflection images for each of the 5 male bystanders from Experiment 1, plus author RJ (6 images in total), were used to construct a lineup-style array, which was presented onscreen at 204 pixels high ×745 pixels wide (see Figure 6). Array items were arranged in different random orders for different participants.

Participants. Ten new volunteers (2 female, 8 male; mean age 36.9) who were naive to the purpose of the experiment participated. All of these participants were *familiar* with the face of RJ (mean acquaintance 18.2 years), and *unfamiliar* with the faces of the bystanders (zero acquaintance; none had visited the University of Glasgow where the bystanders studied).

Procedure. The face array was presented to participants with the following printed task instructions:

> "For any face that you can identify, please write in the person's name. Please also indicate your confidence in each decision (i.e. whether or not you know each face) by providing a confidence rating on a scale of 1 to 10 (1 = guessing, 10 = completely certain). This is not a trick question, we are just trying to establish how useful images like this might be."

No time limit was imposed for completing this task.

Results

Correct naming of the *familiar* face was frequent (hits 90%), and mistaken identification of the *unfamiliar* faces was infrequent (false positives 10%). In addition, confidence ratings were higher for hits (M = 7.89, SD = 1.36) than for false positives (M = 4.80, SD = 3.11), though false positives were too infrequent to allow statistical analysis (n = 5).

Discussion

By zooming in on high-resolution passport-style photographs, we were able to recover images of bystanders from reflections in the eyes of photographic subjects. Performance in the face matching task (Experiment 1) and the spontaneous recognition task (Experiment 2) indicate that these bystander images were not merely *informative* about facial appearance, they were properly *identifiable* to viewers who knew the faces. This is perhaps a surprising result, given the very unpromising source of these images. However, it is consistent with previous evidence that familiar face recognition is extremely tolerant of poor image quality [16]. We note that the reflection images also contain cues to bystanders' emotional state and interest, via facial expression [17], gaze direction [18], and posture [19], although we did not explore those cues here.

One possible extension of this technique would be to combine pairs of images recovered from the subject's two eyes. In principle, these images contain the stereo disparity information required to reconstruct a 3D representation of the environment from the viewpoint the photographic subject [20]. Since corneal reflections extend beyond the aperture of the pupil, such reconstructions could capture a wider angle of the scene than was visible to the subject at the time (see [21] for a related technique).

For now, our findings suggest a novel application of high-resolution photography: for crimes in which victims are photographed, corneal image analysis could be useful for identifying perpetrators. As with other sources of forensic evidence (e.g. fingerprints), corneal reflection images may not always be readily available. In particular, clear corneal reflections require the subject's face to be in focus, and viewed from a roughly frontal angle under good lighting. They also require high image resolution in order for bystanders' faces to be properly resolved. We note that pixel count per dollar for digital cameras has been doubling

approximately every twelve months [22 23]. This trajectory implies that mobile phones could soon carry >39 megapixel cameras routinely. However, as the current study emphasizes, the extracted face images need not be of high quality in order to be identifiable. For this reason, obtaining optimal viewers - those who are familiar with the faces concerned - may be more important than obtaining optimal images.

Supporting Information

Movie S1 Animated zoom on the cornea of a high-resolution photographic subject. The zoom begins with a passport photo-style framing of the subject, and ends with a full face close-up of a bystander captured in the subject's corneal reflection. Successive movie frames represent a linear magnification of 6%. Each frame was resized to 720 pixels wide ×540 pixels high using bicubic interpolation to reduce high spatial frequency noise. Contrast was enhanced separately for each frame using the Auto Contrast function in Adobe Photoshop to improve definition. The image sequence was then converted to movie format for viewing.
(AVI)

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Author Contributions

Conceived and designed the experiments: RJ. Performed the experiments: CK RJ. Analyzed the data: CK RJ. Contributed reagents/materials/analysis tools: RJ. Wrote the paper: RJ.

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months (severe: 0 months, 0 of 50; 12 months, 1 of 46 [2.2%]; moderate: 0 months, 3 of 50 [6.0%]; 12 months, 4 of 38 [10.5%]; $P > .99$). There were no participants with osteoporosis (defined as a *T*-score of −2.5 or less)[46] at 0 or 12 months in the severe or moderate groups.

Fat Mass and Distribution

Differences between the 2 groups in waist and hip circumference, or the ratio thereof, were observed starting at 1 to 4 months. Indeed, the severe group had significantly lower waist and hip circumferences compared with the moderate group at all points after baseline (estimated marginal mean of waist circumference at 12 months: −14.3 [95% CI, −17.3 to −11.3] cm vs −6.9 [95% CI, −10.1 to −3.7] cm; $P < .001$; hip circumference at 12 months: −10.3 [95% CI, −12.7 to −7.8] cm vs −6.2 [95% CI, −8.9 to −3.5] cm; $P < .001$) (Figure 3E and Table 2), and the severe group had significantly lower values of waist to hip ratio than the moderate group at 4, 6, and 12 months, but not at 0.25 or 1 month (estimated marginal mean at 12 months: −0.038 [95% CI, −0.075 to −0.001] vs −0.005 [95% CI, −0.046 to 0.035]; $P < .001$) (Table 2). Both groups had significant decreases in waist and hip circumference at all points compared with baseline, except at 0.25 months for the moderate group. The severe group but not the moderate group had significant reductions in the ratio of waist to hip circumference at 12 months compared with baseline. Moreover, compared with the moderate group, the severe group had significantly lower whole-body fat mass (effect size, −5.5 kg; 95% CI, −7.1 to −3.9 kg; estimated marginal mean at 12 months: −10.2 [95% CI, −12.1 to −8.4] kg vs −5.5 [95% CI, −7.5 to −3.4] kg; $P < .001$) (Figure 3F and Table 2), abdominal subcutaneous and visceral adipose tissue volumes (subcutaneous adipose tissue: effect size, −1890 cm^3; 95% CI, −2560 to −1219 cm^3; estimated marginal mean at 12 months, −3391 [95% CI, −4220 to −2562] cm^3 vs −1624 [95% CI, −2511 to −736] cm^3; $P < .001$; visceral adipose tissue: effect size, −1389 cm^3; 95% CI, −1748 to −1030 cm^3; estimated marginal mean at 12 months, −2379 [95% CI, −2839 to −1919] cm^3 vs −1077 [95% CI, −1561 to −594] cm^3; $P < .001$), intrahepatic lipid (geometric mean ratio at 12 months: 0.32 [95% CI, 0.22 to 0.46] vs 0.40 [95% CI, 0.27 to 0.61]; $P = .04$), thigh subcutaneous adipose tissue area (estimated marginal mean at 12 months: −38.9 [95% CI, −48.6 to −29.2] cm^2 vs −24.0 [95% CI, −34.2 to −13.8] cm^2; $P < .001$) as well as thigh subfascial fat area (geometric mean ratio at 12 months: 0.79 [95% CI, 0.73 to 0.85] vs 0.89 [95% CI, 0.82 to 0.96]; $P < .001$) and intermuscular fat area (estimated marginal mean at 12 months: −1.80 [95% CI, −2.31 to −1.29] cm^2 vs −1.05 [95% CI, −1.58 to −0.51] cm^2; $P < .001$) at all points after baseline (Table 2; eFigure, C-H in Supplement 2). Both groups had significant decreases from baseline in all these parameters.

Adverse Events

There were 8 adverse events (6 in the severe group, all related or possibly related to the intervention, and 2 in the moderate group, neither related to the intervention). These adverse events were reported by participants to the research team or occurred in the clinic during the clinical testing day. No adverse event was considered serious. In the severe intervention, the adverse events were hemorrhoids (2 participants [4.0%]), gallstones (2 participants [4.0%]), and hair loss (2 participants [4.0%]). In the moderate intervention, there were 2 episodes of migraine (1 participant [2.0%]), probably precipitated by fasting prior to clinical testing. This participant had a history of migraines and continued with the trial but only underwent outcome measurements that did not require fasting.

Discussion

d1 This randomized clinical trial demonstrated that, compared with moderate energy restriction over a 12-month period, severe energy restriction resulted in the following: (1) approximately 1.5 times as much loss of whole-body lean mass and thigh muscle area, although these losses were proportional to the amount of weight lost; (2) no difference in handgrip strength; (3) approximately twice as much weight loss (with participants 2.5-3 times more likely to lose 10% of their initial weight); (4) approximately twice as much total fat loss; and (5) a healthier fat distribution, as indicated by

approximately twice the loss of waist circumference, waist-to-hip circumference ratio, and abdominal adipose tissue volume (subcutaneous and visceral). Participants in the severely energy-restricted intervention were also 3 times less likely to discontinue the trial compared with those in the moderately energy-restricted intervention. This is possibly because the large and rapid weight loss associated with severe energy restriction has been shown to be encouraging and because the total meal replacement diet used to achieve it is simple and convenient.[47] These striking findings were offset by an approximately 2.5-fold greater loss of total hip BMD with severe energy restriction compared with moderate energy restriction, a difference not accounted for by the greater weight loss.

In this trial, the participants in the moderate group experienced approximately 1.3% reduction in total hip BMD after 12 months, similar to the annual rate of BMD loss at the hip in the early postmenopausal years (approximately 1.0%-1.4%).[48] However, after the 12-month severe intervention, total hip BMD loss (approximately 3.3%) was 2.4 to 3.3 times higher than these annual BMD losses. Interestingly, the decrease in BMD continued over the whole 12 months of the severe intervention, even though weight loss had plateaued by 6 months. This occurred despite a dietary protein prescription of 1 g/kg of actual body weight per day in both groups[31,49] and despite the fact that the total meal replacement products used in this trial contained more than the Australian recommended dietary intake for vitamin D and calcium for women aged 51 to 70 years.[33] This loss of BMD may have been exacerbated because of the population selected for this trial (women with obesity who were ≥5 years postmenopausal). For example, in a study where men and women with obesity were prescribed a total meal replacement diet until they reached 15% weight reduction in 3 to 6 months, all participant groups exhibited significant decreases in body weight at 2 years, and both groups showed loss of BMD over 2 years, but this loss was statistically significant only among women.[50] This suggests that women may have a greater propensity to lose BMD following weight loss. Research also suggests that BMD loss may be exacerbated in postmenopausal women (as in this trial) compared with women who are still in the perimenopausal transition.[48]

The consequences of accelerated BMD loss with a severely energy-restricted dietary obesity treatment are clinically concerning, especially if BMD loss continues beyond the 12-month intervention, because it has been linked to an increased risk of osteoporosis and fragility fracture.[51,52] However, this bone loss must be considered in light of the beneficial effects of substantial weight loss on other health outcomes and health care costs. For example, although a 3% to 5% loss of initial weight has generally been accepted as being clinically significant,[11,53] recent research shows that greater weight losses, ie, of 7.7%, 10%, 15%, or 20%, dose-dependently improve health outcomes.[54-57] In addition, if treated effectively, the costs of obesity-related health complications would be significantly reduced.[2] Thus, implementing effective obesity treatments is essential to reducing obesity-related comorbidities and the associated costs. Thus, while the current trial should not discourage the use of total meal replacement diets as a treatment for obesity in postmenopausal women, further investigation is needed to determine the long-term consequences of the associated BMD loss on health outcomes such as osteoporotic fractures and to determine how BMD losses could be prevented in this population during and after these diets.

Strengths and Limitations

Strengths of this study include the 12-month randomized clinical trial design and the criterion-standard techniques used for the assessment of body composition, notably our primary outcome of lean mass. Another strength is that data variability was reduced by analysis of all data by a single researcher (ie, S.M. for DXA, A.L.W.-T. for MRI, and S.E.K. for MRS). There are, however, some limitations that must be noted, 1 of which is the technical limitation of DXA, which can only measure 2 tissue types at any time (eg, bone and soft tissue). Thus, being a 2-compartment model for the determination of body composition, a possible confounder is that the DXA analysis assumes a constant hydration of lean soft tissue, which is not always true, as hydration varies with age, sex, and disease. To help control for this, all participants were measured after an overnight fast (≥8 hours)

and after voiding their bladder once at home before attending our clinical research facility and again when they arrived at the clinic. Another technical limitation of DXA is that changes in BMD observed with large weight losses might be exaggerated because of the varying amounts of soft tissue over time, which can result in unpredictable errors in DXA bone measurements, to up to 20%.[58] In addition, DXA assesses bone quantity and not bone quality (eg, bone microarchitecture) or osteoporotic fracture incidence, and it is possible that, despite BMD loss, bone quality and strength may have been preserved in our participants. Despite these technical limitations, DXA remains the criterion standard and the only available test for measuring BMD in clinical practice as well as being an important predictor of osteoporotic fracture.[59,60] A further limitation of our trial is that participants were predominantly white, which limits the generalizability of the findings to populations of other races.

Conclusions

In this randomized clinical trial, severe energy restriction with a total meal replacement diet in postmenopausal women with obesity induced greater weight loss and approximately 1.5-fold as much loss of whole-body lean mass and thigh muscle area compared with moderate energy restriction over 12 months. While these losses of lean tissues were proportional to the amount of weight lost and while muscle strength (ie, handgrip strength) was unaffected by severe vs moderate energy restriction, there was an approximately 2.5-fold greater loss of total hip BMD with severe compared with moderate energy restriction, a difference not accounted for by the greater weight loss. Therefore, caution is necessary when implementing severe energy restriction in postmenopausal women with obesity, especially in those with osteopenia or osteoporosis, for whom concurrent bone-strengthening treatments (eg, muscle strengthening exercises) are recommended.

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