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Integrating muscle cell biochemistry and whole-body physiology in humans: ^{31}P-MRS data from the InSight trial

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We acquired ^{31}P-MRS data from skeletal muscle of subjects of mixed gender and ethnicity, combined with a panel of physiological characteristics, and tested several long-standing hypotheses regarding relationships between muscle cell biochemistry and whole-body physiology with unusually high statistical power. We hypothesized that i) whole-body VO_2max would correlate with muscle respiratory capacity, ii) resting muscle phosphocreatine concentration ([PCr]) would negatively correlate with delta efficiency and iii) muscle mitochondrial function would positively correlate with both resting VO_2 and total daily energy expenditure (TDEE). Muscle respiratory capacity explained a quarter of the variation in VO_2max ($r^2 = 26$, $p < .001$, $n = 87$). There was an inverse correlation between muscle [PCr] and delta efficiency ($r = -23$, p = 046, $n = 87$). There was also a correlation between [PCr] recovery halftime and TDEE (r = −23, p = 035, $n = 87$). Our data not only provide insights into muscle cell chemistry and whole-body physiology but our mixed cohort means that our findings are broadly generalizable.

Nuclear magnetic resonance spectroscopy (MRS) offers the opportunity to study the relationships between cell metabolism and whole-body physiology by providing noninvasive, quantitative, temporally- and spatially-resolved measurements of cell biochemistry *in vivo*. This has been especially true of skeletal muscle, where 31-phosphorus (^{31}P)-MRS has been much used, in living humans and animals, to measure phosphorus biochemistry, energy metabolism, mitochondrial function, proton handling and contractile efficiency (among others)[1]. As biology enters an era in which both reductionist and systems approaches are connected by studies of *in vivo* function, MRS continues to play an important role in understanding the relationships between cell/tissue biology and whole-body physiology – i.e. integrating cell, organ and organism.

As part of a large prospective longitudinal study (InSight), we acquired ^{31}P-MRS data from skeletal muscle in a large number of subjects (~90), in parallel with an extensive panel of important physiological and morphological characteristics including maximal whole-body respiratory capacity (VO_2max), body composition and muscle performance. This provided an opportunity to study the relationships between *in vivo* muscle chemistry, muscle mitochondrial function and whole-body physiology with high statistical power. Furthermore, the cohort involved in this trial, while young, was mixed in both gender and ethnicity. Therefore any relationships that might be uncovered should be more broadly generalizable than those from existing studies that have been conducted in more homogeneous cohorts. Nevertheless, our results are still restricted to subjects who are young and relatively fit and should not be extended beyond these.

We made a number of hypotheses before analyzing the data, based on current evidence regarding how muscle cell biology translates upwards into physiological function. These are summarized in Table 1, with associated references. The meaning of the symbols, and the methods used, are described in Methods. We also used untargeted correlations and an unsupervised multivariate statistical method (principal components analysis) for data mining.

Table 1 | Predicted correlations between ^{31}P-MRS measures of skeletal muscle biology and whole-body physiology

x	y	Direction	Hypothesis	Refs	Hypothesis supported?
$PCr_{1/2t}$	VO_2max	—	Mitochondrial function will positively correlate with both	21,23	Yes ($p < .0001$)
Qmax, ATPmax	VO_2max	+	absolute and mass-corrected VO_2max		
Resting [PCr]	Delta efficiency	—	Resting muscle [PCr] will negatively correlate with delta efficiency	2,26,34	Yes ($p = .046$)
$PCr_{1/2t}$	Resting VO_2, TDEE	—	Mitochondrial function will positively correlate with both	31	TDEE: Yes ($p = .032$)
Qmax, ATPmax	Resting VO_2, TDEE	+	resting VO_2 and total daily energy expenditure		VO_2: No

In addition to the study of relationships between muscle and whole-body physiology, the InSight data allowed us to report reference values and ranges for several important phosphorus metabolites and metabolite ratios in a young, disease-free, racially and gender-mixed cohort, which will be of general use for assessing individual patients' data. Finally, we tested for potentially important differences between groups that would need to be accounted for in future studies.

Results

Descriptive data. Our subjects ($n = 89$) comprised 39 males and 50 females. They had a median age of 26 years (interquartile range (IQR) = 8 years) and their median mass-corrected maximal aerobic capacity was 36 mL kg^{-1} min^{-1} (IQR = 13 mL kg^{-1} min^{-1}). Their BMI (separated by gender) was 23.4 ± 0.4 kg m^{-2} (males) and 22.3 ± 0.3 kg m^{-2} (females). Fasting glucose (GLU) was 86 ± 1 mg/dl (4.78 ± 0.06 mmol/l) and fasting insulin (INS) was (median ± IQR) 3.9 ± 3.3 μU/mL.

Normal values and ranges for resting quadriceps muscle phosphorus metabolites. Table 2 gives the means and standard errors for each phosphorus metabolite that was directly measured. We did not attempt absolute quantification, so the values are given in two forms –a ratio relative to the γ-phosphorus of ATP, and as a millimolar concentration based on an assumed concentration of ATP in (see Methods).

Resting muscle pH was 7.07 ± 0.003. We also calculated several indices of muscle respiratory capacity. Although these would somewhat depend upon the exact protocol used, the means for each were: $PCr_{1/2t}$ = 21.9 ± 0.6 s; k = 0.034 ± 0.001; ATPmax = 1.13 ± 0.03 mM s^{-1}; Qmax = 0.70 ± 0.03 mM s^{-1}.

We tested for differences in all these values by both gender and ethnicity, to highlight important physiological differences that may need to be accounted for in future studies. The only significant difference by group was resting muscle pH between genders: Male (n = 39): 7.09 ±.004, vs. Female (n = 50): 7.06 ±.003; $p < 10^{-7}$.

Predicted relationships. As expected, there were significant correlations between all MR measures of *in vivo* muscle mitochondrial function and both relative (mass-corrected) and absolute VO_2max (Figure 1).

Interestingly, the respiratory exchange ratio (RER) at VO_2max (an indicator of the contribution of glycolytic metabolism at maximal exercise) and the initial rate of PCr resynthesis after cessation of plantar flexion exercise (an indicator of the contribution of oxidative metabolism at the end of plantar flexion exercise) were inversely correlated ($r = -.25$, $p = .023$, $n = 86$), showing a link between the contributions of various metabolic pathways in muscle and at the whole-body level.

Table 2 | Mean values of phosphorus metabolite ratios and concentrations in a young, ethnically-diverse and gender-mixed cohort. ($n = 89$)

Metabolite	Relative to γ-ATP	mM
PCr	4.1 ± 0.03	33 ± 0.3
Pi	0.47 ± 0.007	3.9 ± 0.06
PME	0.58 ± 0.05	4.8 ± 0.4
PDE	0.76 ± 0.05	6.2 ± 0.4
NADP	0.12 ± 0.01	1.0 ± 0.08

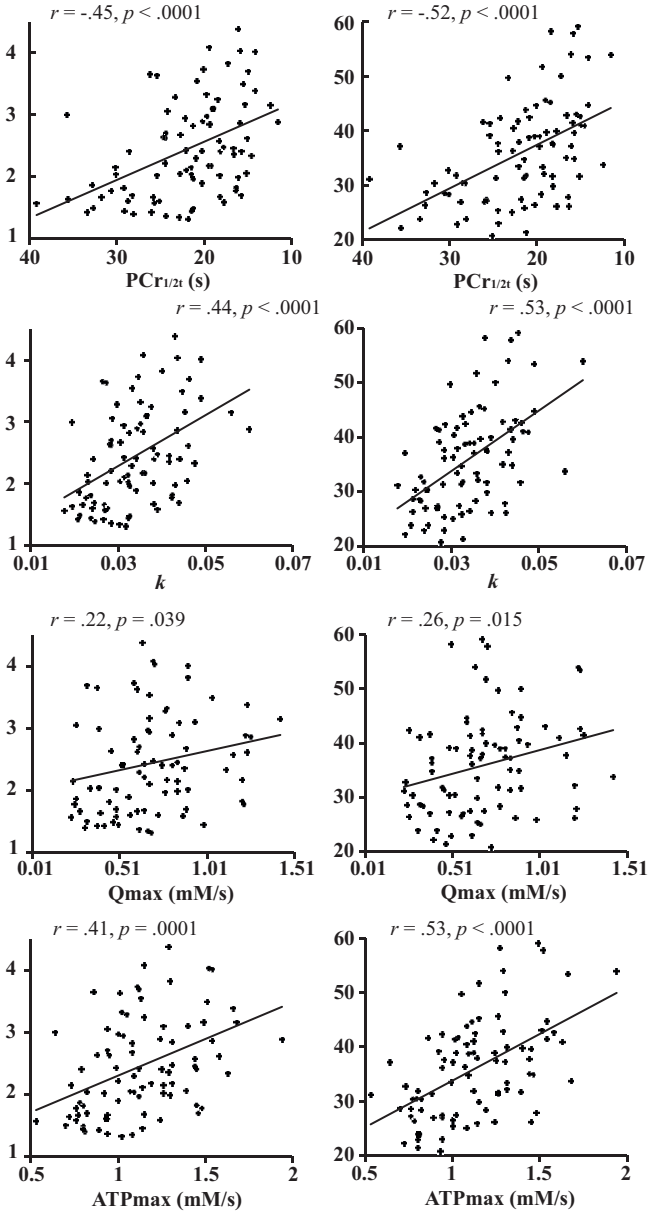

Figure 1 | Correlations between ^{31}P-MRS estimates of mitochondrial function and either absolute (left column) or mass-adjusted (right column) VO_2max ($n = 86$). Absolute VO_2max is in L min^{-1}, mass-adjusted VO2max is in mL min^{-1} kg^{-1}.

We had predicted that, due to the relationship between resting muscle phosphocreatine (PCr) content and muscle fibre-type distribution, we would observe an inverse correlation between [PCr] and delta efficiency (DE). This was found, with resting [PCr] significantly inversely correlated with DE ($r = .22$, $p = .046$, $n = 87$). Although DE did not correlate with Qmax or ATPmax, it was significantly correlated with k ($r = .23$, $p = .037$, $n = 86$) and negatively correlated with $PCr_{1/2t}$ ($r = -.29$, $p = .006$, $n = 86$), suggesting that better mitochondrial function was associated with higher delta efficiency.

Total daily energy expenditure (TDEE), but not resting oxygen uptake, was correlated with measures of muscle mitochondrial function. Both k ($r = -.22$, $p = .038$, $n = 86$) and $PCr_{1/2t}$ ($r = -.23$, $p = .032$, $n = 86$) were significantly related to TDEE, signifying a positive relationship between muscle mitochondrial function and daily energy expenditure (Figure 2).

Data mining – untargeted correlations. There were a number of unpredicted and highly significant ($p < 10^{-4}$) correlations between Biodex measures of muscle strength and intramuscular pH. On closer examination, these were the result of the previously observed difference in muscle pH between genders, highlighting the importance of accounting for this difference in future studies. In addition, there were two significant correlations between MR measures of muscle chemistry and muscle performance. First, there was a significant correlation between muscle PCr/Pi and average power during the five 60 deg s^{-1} leg extensions ($r = -.29$, $p = .008$). Second, there were significant correlations between all measures of mitochondrial function and power output during the last five extensions of the fatigue index protocol (for example, $PCr_{1/2t}$: $r = -.38$, $p = .0004$).

We performed 63 pairwise correlations of anthropometry vs. MRS data (Anthropometry: waist, waist/hip, weight, BMI, percent body fat, fat mass and lean mass (seven variables); vs. MRS: PCr, Pi, ADP, PME, PDE, NADP, $PCr_{1/2t}$, ATPmax and Qmax (nine variables)). These variables are clearly not independent, making assessment of significance problematic, although both Bonferroni and Sidak corrections suggest an adjusted significance threshold of approximately $p < .001$. There were correlations between all measures of mitochondrial function and both percent body fat and fat mass (Figure 3). Although no single correlation had $p < .001$, several were borderline ($p = .003-.004$) and, taken together, these correlations provided persuasive evidence of a link between fatness and mitochondrial function. There were no other significant correlations between any measures of anthropometry or body composition and muscle biochemistry or mitochondrial function. Likewise, there were no significant correlations between any measured plasma metabolite (HDL, LDL, TAG, CHOL, GLU, INS) and muscle phosphorus chemistry or mitochondrial function.

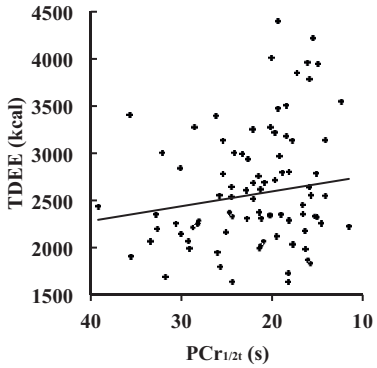

Figure 2 | Correlation between $PCr_{1/2t}$ (see main text for details) and total daily energy expenditure (TDEE); relationship is significant at $p < .05$.

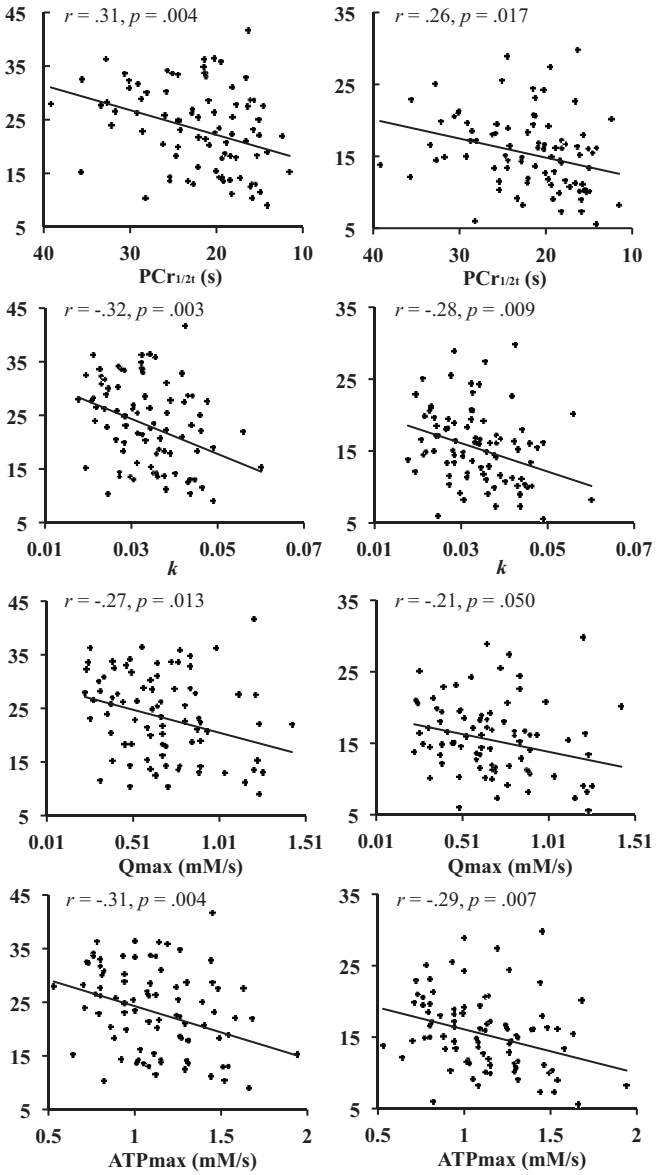

Figure 3 | Correlations between ^{31}P-MRS estimates of mitochondrial function and percent body fat (left column) or fat mass (right column, in kg) ($n = 86$).

Data mining – principal components analysis. In order to look for unpredicted patterns in the ^{31}P-MRS data we performed principal components analysis (PCA) on the known components of the resting muscle spectra. Two principal components (PC1 and PC2) were sufficient to explain 84% of the variance in the data, suggesting substantial collinearity between phosphorus resonances. This collinearity was not due to resonances from the same metabolite (for example, the expected correlation between the resonances from the α, β and γ phosphorus nuclei of ATP). In particular, the loading coefficients for PC1 showed significant collinearity between phosphocreatine and phospho- di- and mono-esters. However, as can be seen from Figure 4, there was no obvious clustering or patterning in the dimensionally-reduced data.

Discussion

Since a period of rapid technical discovery and development in the 1980s, nuclear MRS has provided an unrivalled tool to study the biochemistry (and biophysics) of living cells in real time. Now that the reconciliation of cell and tissue data with whole organism

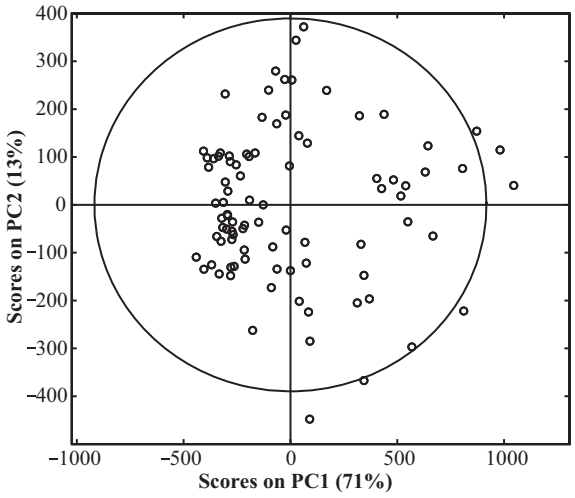

Figure 4 | Principle components analysis scores biplot for ^{31}P-MRS data from 87 subjects of mixed gender and ethnicity.

physiology is becoming a priority, MR will continue to offer unique advantages over more destructive methods (mass spectrometry) or those that can only be used under very specific circumstances (live cell imaging). In this paper, we report our results from an unusual data set – combined ^{31}P MRS and applied physiology measurements made on a particularly large cohort of healthy humans of mixed ethnicity and gender. As a result, we were able to test a number of hypotheses relating muscle biochemistry to whole body physiology in normal humans with unusually high statistical power.

In general we found that muscle biochemistry was reflected in whole body physiology in a manner that was consistent with existing theory and data, and with our *a priori* hypotheses. For example, based on earlier findings in smaller, more specialized cohorts[21–23] we predicted that, in a large cohort of mixed gender and ethnicity, whole-body VO_2max would correlate with muscle respiratory capacity, measured using ^{31}P-MRS. Our hypothesis was supported: $PCr_{1/2t}$ (negatively) and Qmax and ATPmax (positively) correlated with both absolute and mass-corrected VO_2max (Figure 1). Although the correlation coefficients appear low this supports the notion that VO_2max is a complex measure of integrative physiology that is only partly determined by mitochondrial function. Indeed, the magnitude of these relationships suggested that approximately 20–25% of the variation in mass-corrected VO_2max could be explained by variations in muscle respiratory capacity, a finding that was also consistent with previous work. The remainder of the variation in VO_2max was presumably due to extra-muscular physiological factors such as diffusive and/or convective oxygen delivery (and possibly the neuropsychological factor of motivation). The halftime of PCr recovery appeared to be a more robust indicator of VO_2max than either of the extrapolated/calculated values (Qmax or ATPmax), while ATPmax was a better predictor of both absolute and mass-adjusted VO_2max than was Qmax. This may however simply reflect the greater mathematical/theoretical simplicity of ATPmax, resulting in reduced noise (but not necessarily better reflecting underling physiology).

Delta efficiency during cycling is related to the percentage of oxidative fibres in the locomotor muscles[2]. Given that PCr concentration in oxidative fibres is lower than in glycolytic fibres[24] and therefore mean PCr concentration in mixed muscle reflects fibre type distribution[15,25,26], we tested the hypothesis that muscle PCr content, measured using ^{31}P-MRS, would be positively correlated with DE (the slope of VO_2 against work rate at work rates below VT[27]) during incremental exercise testing. In this case our hypothesis was supported by the data ($r = .22$, $p = .046$), strengthening not only the earlier (but disputed[28]) findings of Coyle et al.[14], but also the utility of [PCr] as an index of muscle fibre type distribution between individuals.

There is an ongoing debate as to whether muscle mitochondrial function and delta efficiency are related (cf the introduction in[29]). For example, Lucia *et al.* reported an inverse relationship between VO_2max and efficiency (during a steady-state exercise task)[30] suggesting an inverse relationship between muscle oxidative capacity and delta efficiency. However, this study was carried out in a highly-selective group (world-class endurance cyclists), so the results may have reflected not 'normal' physiology but the result of a selective process within tight constraints - those with marginal aerobic capacity may have been nevertheless able to succeed as professional road cyclists precisely because they were highly efficient. Thus we decided to test for a relationship between delta efficiency (as defined above) and muscle respiratory capacity. We found a significant inverse correlation between $PCr_{1/2t}$ and DE ($r = -.29$, $p = .006$), such that better mitochondrial function was associated with higher delta efficiency. Our findings are at odds with those of Hunter *et al.* ([29]), who reported an inverse correlation between exercise economy (defined as the oxygen cost of a given walking velocity during steady-state walking) and muscle respiratory capacity (measured, as here, using ^{31}P-MRS). However, these disparate results might be reconciled by differences in both methodology (DE compared with VO_2 during steady state walking) and cohort (pre-menopausal women only, compared with a mixed gender and ethnicity cohort). Given that we also found a positive correlation between PCr concentration and delta efficiency, it is tempting to suggest that both correlations ([PCr] vs. DE and $PCr_{1/2t}$ vs. DE) are the result of underlying differences in fibre type distribution. Unfortunately it is a limitation of the present study that we did not measure fibre type distribution directly.

Given an earlier finding that resting metabolic rate was positively related to muscle ATP-synthase content[31], we decided to test our hypothesis that resting metabolic rate was related to muscle respiratory capacity. The data supported our hypothesis: $PCr_{1/2t}$ was significantly related to total daily energy expenditure (TDEE) ($r = -.23$, $p = .030$) (Figure 2). Therefore those with higher muscle respiratory capacity had increased daily energy expenditure. The nature of causality between these variables is, as yet, unclear. One might reasonably hypothesize that increased daily activity led to improved muscle respiratory capacity and this view is somewhat supported by the absence of a positive relationship between resting VO_2 and muscle oxidative capacity.

Intriguingly, we found good evidence linking body composition to muscle mitochondrial function. Again, the direction of causality here is unclear. One would expect those who are more active to use more energy and therefore to have higher muscle (and whole-body) respiratory capacities and less body fat. By Occam's razor, this explanation would seem to be more likely than the concept that increased fat mass was somehow inhibiting muscle mitochondrial function However, there was a very significant relationship between resting oxygen uptake and TDEE ($r = .43$, $n = 86$, $p = 0.00003$), such that one might also reasonably argue that some subjects were genetically predisposed to having a higher resting energy cost, better muscle mitochondrial function and therefore had a higher TDEE and lower fat mass.

There were no consistent differences in muscle biochemistry between genders or by ethnicity, with one exception. Muscle pH was significantly lower in females compared with males (7.06 $\pm$.003 vs. 7.09 $\pm$.004, $p < 10^{-7}$). Although this appears at first to be only a minor difference it amounts to a 7% higher proton concentration in males. The determinants of intracellular pH setpoint in themselves remain far less well understood in skeletal muscle than in cardiac muscle[32], although the principle is that basal pH is determined by the steady state balance between a number of sarcolemmal

proton exchangers and uniporters. Furthermore the significance of this pH difference by gender is difficult to determine, although it must be considered if comparing any pH-dependent variable between groups that are unbalanced for gender. Beyond this, our unsupervised multivariate analysis of the resting spectra revealed no unexpected grouping.

This dataset represented a unique opportunity to study relationships between non-invasive measures of muscle biochemistry with whole-body exercise-physiology, with particular reference to oxidative metabolism, in a group of normal subjects large enough to establish these with high statistical power. Unlike earlier studies in this area we did not study the effects of formal exercise training, and especially not adaptions in professional athletes where, as noted in the Discussion, selection effects can give apparently paradoxical results. The results broadly confirmed our pre-specified hypotheses that a substantial fraction of variability in whole body aerobic fitness could be explained by variations in muscle mitochondrial function, and that [PCr] correlated with delta efficiency in a way consistent with its being a surrogate for average muscle fibre type composition. We also found that muscle mitochondrial function was a positive predictor of both daily energy expenditure and leanness. Finally, we found that there was a previously unreported gender difference in resting muscle pH. These findings are important for those with an interest in the genetic and physiological determinants of whole-body aerobic capacity, as well as those with broader interests in human physiology and health (given that VO_2max is a strong predictor of all-cause mortality[33]).

Methods

Ethical approval. This study is a registered clinical trial (ClinicalTrials.gov identifier:NCT00945633) and was approved by the ethical review board of the Pennington Biomedical Research Institute. Written informed consent was obtained from all participants prior to the study, which conforms to the guidelines in the Declaration of Helsinki.

Participants. In brief, the InSight study is a prospective, longitudinal, clinical study using an epidemiological approach to identify dietary, physiological, genetic and behavioral determinants of unhealthy weight gain over a ten-year period. Inclusion criteria for the InSight study were as follows: men and women aged 20–35 y, BMI $<$ 27.5 kg m^{-2}, fasting blood glucose $<$ 126 mg/dl (7.0 mmol L^{-1}); clinical and physical examination to confirm health prior to acceptance (exclusion criteria detailed below). Participants were 50% black and 50% Caucasian with an equal gender mix within each ethnic group. The design was intended to allow the identification of subtle differences in physiology and behavior that tracked along the level of familial risk.

Exclusion criteria included the following: history of diabetes, history of obesity (BMI $\geq$ 30 kg/m^2), history of known inherited medical conditions that might influence future health status, current or planned medication usage that might influence future health status, prior serious injuries/surgeries that might influence future health status, women who were pregnant or breastfeeding at recruitment (pregnancy did not cause already-enrolled subjects to be removed from the study, nor were pregnancy or childbirth considered adverse events), women who were $<$ 6 months post-partum, or women who had discontinued breastfeeding $<$ 3 months prior to screening, history of cancer (including skin cancer) within 5 years, history of organ transplant, previous diagnosis with HIV, Hepatitis B or C, or tuberculosis, abuse of alcohol or illegal drugs, abnormal ECG, presence of a pacemaker, defibrillator, or implanted metal, history of eating disorders and abnormal psychological scores at screening.

Participants entering the InSight study were initially screened over 3 visits to the Pennington Biomedical Research Center (PBRC) in order to determine eligibility and obtain height, weight, waist and hip measurements, vital signs, a resting ECG, a medical history and physical examination, lab work (full blood count and standard blood chemistry (high-density lipoproteins (HDL), low-density lipoproteins (LDL), triacylglycerols (TAG), cholesterol (CHOL), fasting glucose (GLU) and fasting insulin (INS)), urinalysis, urine drug screen) and the completion of various questionnaires, including a PBRC Screening Health Questionnaire and demographic questionnaire. During these visits the participants were assessed on a range of skeletal muscle and whole-body characteristics including cardiorespiratory fitness (VO_{2max}), muscle strength and performance, anthropometry and ^{31}P-MRS measures of muscle chemistry and metabolism (in 89 participants), the methods for which are described below.

Anthropometry. We measured body mass in a gown after voiding and waist circumference using a standardized protocol. Height was measured on a calibrated stadiometer. Percent body fat, fat mass and lean mass were measured on a Hologic Dual Energy X-ray Absorptiometer (DXA, QDR 4500A, Hologic, Inc., Waltham, MA.) During the DXA, scan, participants were asked to lie on a table wearing a hospital gown for 4–6 minutes during the scanning process. Two distinct energies were used to determine body mineral and soft tissue content. An attenuation ratio was determined from a known tissue content. Variations of the attenuation ratio determined the fat content of the tissue at each pixel thereby calculating the percentage body fat. The scans were analyzed with QDR software for Windows V11.1. The coefficient of variation (CV) for the body composition measurement of lean mass, fat mass and percentage body fat was 0.8%, 1.6%, and 1.7%, respectively.

Exercise testing. *Maximal cardiorespiratory testing.* We performed all maximal cardiorespiratory exercise testing (VO2max) in the PBRC Exercise Biology Exercise Testing Core. During the VO_{2max} test, we monitored each participant using a 12-lead electrocardiogram (Q-Stress, Quinton Instruments, Seattle, WA). All exercise tests were performed using a standardized graded exercise testing protocol administered on a treadmill (Trackmaster 425, Newton, KS). The participants performed a brief, self-selected walking speed as a warm-up, then the test was initiated a speed of 1.7 mph and 1% grade. Each testing stage lasted two minutes and speed and/or grade were increased every two minutes until the participant reached a state of temporary exhaustion and could no longer continue the test. The determination of VO_2max required the achievement of two of the three following conditions:

(1) a plateau or rise in VO_2 (L min^{-1}) $<$ 100 mL,
(2) RER $>$ 1.1, and
(3) a maximal heart rate within 10 beats/min of the participants age predicted maximum.

During the testing all ventilatory measures were collected breath-by-breath and then averaged into one minute epochs for later analysis to determine VO_2max and underlying measures of exercise capacity and function. These included maximal heart rate, maximal oxygen uptake (VO_2), pulmonary ventilation (VE), ventilatory equivalents for oxygen (VE/VO_2), carbon dioxide (VE/CO_2), end-tidal partial pressure of oxygen ($PETO_2$) and carbon dioxide ($PETCO_2$), and the calculation of delta efficiency (DE). DE was calculated as the reciprocal of the slope of the relationship between work accomplished per minute and energy expended per minute during the incremental exercise test[2].

Muscular strength and endurance testing. After 5-min of low- to moderate-effort warm-up exercise on a treadmill, concentric isokinetic strength of the knee flexors and knee extensors (right leg) were assessed on a Biodex System 3 dynamometer (Biodex Medical Systems, Shirley, New York). Two tests were performed in order to determine leg strength and endurance: five 60°/second isokinetic knee extensions and flexions, followed by thirty 180°/second isokinetic knee extensions and knee flexion. Each test was preceded by two rehearsal trials using 3–5 repetitions and all tests were interleaved with one-minute recovery periods. Average power, average work and peak torque were then calculated for each test and expressed in absolute terms. We further calculated a muscle fatigue index from the 180°/second isokinetic test that was defined as the percent decrease in peak torque during the 30 repetition set as follows:

Percent decrease = 100−[(mean of the last 5 repetitions)/mean of the highest consecutive 5 repetitions) × 100].

Total daily energy expenditure. Doubly-labelled water (DLW) was used to obtain an accurate measure of total daily energy expenditure (TDEE) and, during a period of energy balance, TDEE is equal to energy intake[3,4]. TDEE was adjusted for changes in body weight following the methods outlined by Schulz et al[5]. Briefly, we assumed that 2/3 of any change in body weight was metabolic and 1/3 was water. In addition, we assumed that 3/4 of this change in metabolic weight was fat mass (FM) and 1/4 was fat-free mass (FFM)[6]. For weight loss, we assumed 9 kcal/g of fat mass (FM) and 1 kcal/g of fat free mass (FFM). For weight gain, we assumed 13.2 kcal/g of FM and 2.2 kcal/g of FFM[7–9]. These methods are consistent with those used in Tataranni et al[10].

Although these methods are commonly used to measure energy intake with DLW when energy balance is not present, the PBRC's Human Physiology Laboratory recently found that correcting TDEE data for change in energy stores results in less accurate measures of energy intake during periods of significant calorie restriction (30%)[11]. Therefore, we used DLW when participants were essentially weight stable. We also closely evaluated changes in energy stores and corrected for minor deviations in energy balance using the methods outlined above. Based on previous research, we are confident that the timing of the DLW measures ensured that we obtained accurate measures of energy intake using DLW.

Participants were given a mixture of 1 part deuterium and 19 parts ^{18}O, followed by a 100-ml of tap water used to rinse the dose container. All subjects received a dose of 1.5 g/kg total body weight. The dose was given early in the morning after an overnight fast and collection of two baseline urine specimens. Participants' body weight was measured and recorded. Two urine specimens were collected and discarded 1.5 hours and 3 hours post dose. A urine specimen was collected 4.5 hours and 6 hours post dose. Body weight also was measured and recorded in the morning on day 7, and a morning urine sample was collected in the clinic (another sample was collected prior to the participant leaving the clinic on day 7). This procedure was repeated on Day 14, along with a DXA scan (see above).

Magnetic resonance spectroscopy protocol. Muscle phosphorus metabolite concentrations, muscle pH and mitochondrial function were determined using a 3T GE Signa MNS magnet (GE, Milwaukee, WI) and ^{31}P-tuned surface coil positioned over the distal vastus lateralis. Following the acquisition of a fully relaxed spectrum, ^{31}P spectra were acquired every 6 seconds at rest and continuously during a 24-, 30- or 36-sec ballistic exercise protocol that comprised 'kicking' against Velcro straps positioned tight across the leg and thigh. To correct for the effects of partial saturation due to the short TR (6 sec) we calculated correction factors from the fully relaxed spectrum and 6-sec spectra acquired from the same volume of resting muscle. Exercise time and intensity was targeted to reduce muscle phosphocreatine (PCr) content by 1/3 to 1/2 from resting values whilst ensuring pH did not fall below 6.8 (to avoid well-known technical complications in interpreting estimates of mitochondrial function in the presence of large pH change[12]).

Magnetic resonance data processing. Spectra were processed using jMRUI version 4[13] and quantified using a non-linear least squares algorithm (AMARES[14]). Resting ATP and total creatine concentrations were assumed to be 8.2 $mM \cdot L^{-1}$ and 42 $mM \cdot L^{-1}$ respectively. These commonly-used concentrations are based on extensive published values, and are reliable in healthy humans[15]. The chemical shift of the inorganic phosphate (Pi) peak relative to phosphocreatine (PCr) (σ, in parts per million) was used to determine intracellular pH. The myocellular free adenosine diphosphate (ADP) concentration was calculated making the standard assumption that that the creatine kinase reaction is at equilibrium, and allowing for changes in pH[16].

Indices of mitochondrial function. We calculated four commonly-used indices of muscle mitochondrial function. The first, the halftime of PCr recovery after moderate exercise ($PCr_{t1/2}$), is an inverse index of *in vivo* mitochondrial function, and was determined by fitting a monexponential equation expressing PCr concentration (normalized to resting values) as a function of time (t), PCr (t) = 1 − exp ($-k\,t$). Microsoft Solver was used to estimate the second – the rate-constant (k) - by least-squares fit, from which $PCr_{t1/2} = \ln(2)/k$[17]. The third, ATPmax, was calculated using the PCr recovery time constant ($\tau = 1/k$) (derived as above) and the concentration of PCr in the same muscle at rest: ATPmax = resting [PCr]/τ[18]. The final index of mitochondrial function, Qmax, is based on the sigmoidal relationship between [ADP] and respiratory rate (V)[19] and is calculated from values for both [ADP] and V at exercise cessation: $Qmax = \{V + (K_m/[ADP])^n\}$, where K_m is the concentration of [ADP] at which respiration is half-maximal (25 μM) and n is a Hill coefficient = 2.2[19]. One subject had values for these estimates of mitochondrial function that were more than five standard deviations from the group mean, and was removed from this part of the analysis.

Statistics. As much of the analysis presented herein relies on measures of correlation, and because Pearson's r is a distribution-free statistic (in other words, it does not rest on the assumption of a normal distribution[20]), we did not routinely check our data for normality. However, in cases where this assumption was made (for example, testing for differences between males and females) the assumption of normality was checked using Shapiro-Wilk and violations were accounted for if necessary.

Where an effect was predicted in advance (for example, the positive relationship between muscle mitochondrial function and whole-body VO_2max), statistical significance was accepted at $p < .05$. However, to lessen the risk of Type I errors when relationships were not predicted in advance, the significance threshold was adjusted as described in the main text, although statistically marginal results were given extra credence if accompanied by a robust biological explanation. Values are reported as mean ± standard error. Data that were not normally-distributed are reported as median ± interquartile range (IQR). All statistical analyses except for principal components analysis (PCA) were conducted using PASW 18.0 (SPSS Inc., Chicago, Illinois, USA). For PCA, the data were processed in AMARES as described above. However, rather than normalize the spectra to the intensity of the γ-ATP resonance we normalized each spectrum by the sum of all peaks (integral-sum normalization). The data were mean-centered and Pareto-scaled before being analyzed using PCA, all in PLS Toolbox 6.2 (Eigenvector Research Inc., Wenatchee, US).

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Author contributions

These experiments were conducted at PBRC. Data analysis was conducted at the University of Tasmania Medical School and the School of Biological Sciences, University of Essex. SRS and CPE conceived and designed of the experiments. LME, GJK, RMD, HF, JTW and CPE collected, analysed and interpreted the data. LME, GJK, RMD, JTW, SRS and CPE drafted the article or revised it critically for important intellectual content.

Additional information

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