Resting skeletal muscle PNPLA2 (ATGL) and CPT1B are associated with peak fat oxidation rates in men and women but do not explain observed sex differences

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Abstract
We explored key proteins involved in fat metabolism that might be associated with peak fat oxidation (PFO) and account for sexual dimorphism in fuel metabolism during exercise. Thirty-six healthy adults [15 women; 40 ± 11 years of age; peak oxygen consumption 42.5 ± 9.5 ml (kg body mass)\textsuperscript{−1} min\textsuperscript{−1}; mean ± SD] completed two exercise tests to determine PFO via indirect calorimetry. Resting adipose tissue and/or skeletal muscle biopsies were obtained to determine the adipose tissue protein content of PLIN1, ABHD5 (CGI-58), LIPE (HSL), PNPLA2 (ATGL), ACSL1, CPT1B and oestrogen receptor α (ER\textsubscript{α}) and the skeletal muscle protein content of FABP 3 (FABPpm), PNPLA2 (ATGL), ACSL1, CTP1B and ESR1 (ER\textsubscript{α}). Moderate strength correlations were found between PFO [in milligrams per kilogram of fat-free mass (FFM) per minute] and the protein content of PNPLA2 (ATGL) [$r_s = 0.41 (0.03–0.68), P < 0.05$] and CPT1B [$r_s = 0.45 (0.09–0.71), P < 0.05$] in skeletal muscle. No other statistically significant bivariate correlations were found consistently. Females had a greater relative PFO than males [7.1 ± 1.9 vs. 4.5 ± 1.3 and 7.3 ± 1.7 vs. 4.8 ± 1.2 mg (kg FFM)\textsuperscript{−1} min\textsuperscript{−1} in the adipose tissue (\textit{n} = 14) and skeletal muscle (\textit{n} = 12) subgroups, respectively (\textit{P} < 0.05)]. No statistically significant sex differences were found in the content of these proteins. The regulation of PFO might involve processes relating to intramyocellular triglyceride hydrolysis and mitochondrial fatty acid transport, and adipose tissue is likely to play a more minor role than muscle. Sex differences in fat metabolism are likely to be attributable to factors other than the resting content of proteins in skeletal muscle and adipose tissue relating to triglyceride hydrolysis and fatty acid transport.

KEYWORDS
adipose tissue, fat metabolism, skeletal muscle

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INTRODUCTION

The oxidation of fatty acids as a fuel source continues to be an area of interest for both human health and exercise performance (Goodpaster & Sparks, 2017; Kiens, 2006). Several regulatory sites are likely to contribute to the oxidation of fatty acids in skeletal muscle during exercise; these include: (i) fatty acid delivery to skeletal muscle; (ii) sarcolemmal translocation of fatty acids; (iii) availability of intramyocellular triglycerides (IMTGs); (iv) cytosolic activation (or transportation) of fatty acids; and (v) mitochondrial factors (Jeppesen & Kiens, 2012; Lundsgaard et al., 2018; Sahlin, 2009). The relative importance of these factors is partly dependent on the exercise conditions. For example, fatty acid delivery is a major factor at low- to moderate-intensity exercise [e.g., below 50% of peak oxygen consumption (\( \dot{V}_\text{O}_2\text{peak} \)], whereas mitochondrial factors [in particular, the regulation of carnitine palmitoyltransferase 1 (CPT1)] are primarily responsible for the reported decline in fat oxidation rates during high-intensity (>70% \( \dot{V}_\text{O}_2\text{peak} \)) exercise (Frayn, 2010; Lundsgaard et al., 2018; Sahlin, 2009; Spriet, 2014).

Previous studies have explored associations between the peak fat oxidation (PFO) rate during exercise (a potential marker of the capacity of an individual to oxidize fat) and factors that reside at the level of skeletal muscle (e.g., skeletal muscle fibre composition and capillary density, mitochondrial content and key enzymes involved in \( \beta \)-oxidation or IMTG hydrolysis) (Dandanell et al., 2018; Haufe et al., 2010; Nordby et al., 2006, 2015; Rosenkilde et al., 2015; Shaw et al., 2020; Stisen et al., 2006). However, no study to date has explored such associations within other key tissues that contribute to fatty acid delivery, such as adipose tissue. Thus, there is a need to expand our knowledge on proteins that might contribute to the regulation of PFO.

One factor that influences fatty acid oxidation is biological sex (Devries, 2016; Lundsgaard et al., 2017). Females typically have a greater PFO [expressed relative to fat-free mass (FFM)] and reliance upon fat as a fuel source during exercise (Devries, 2016; Fletcher et al., 2017; Tarnopolsky, 2008). This sexual dimorphism is attributable to many factors. Firstly, females might have increased fatty acid delivery to skeletal muscle owing to alterations in: (i) adipose tissue lipolytic sensitivity to catecholamines (Horton et al., 2009; Schmidt et al., 2014); (ii) skeletal muscle vasculature (Lundsgaard et al., 2017); and (iii) the content of sarcolemma fatty acid transporters, namely FAT/CD36 (Kiens et al., 2004; Miotto et al., 2018). Secondly, some studies report sex differences in IMTG turnover during exercise, potentially attributable to altered IMTG content (Devries, 2016) and lipid droplet morphology (Devries et al., 2007; Peters et al., 2012; Tarnopolsky et al., 2007). Thirdly, females have an increased capacity for \( \beta \)-oxidation relative to glycolytic flux (Lundsgaard et al., 2017). Lastly, sex differences in concentrations of oestrogen and its actions via oestrogen receptor \( \alpha \) might account for the increase in fat oxidation during exercise in females compared with males (Devries, 2016; Oosthuyse & Bosch, 2012). Nevertheless, these inferences derive from a relatively small body of evidence, and further sex-comparison studies would help to improve our understanding of the mechanisms that underlie sexual dimorphism in exercise metabolism (Lundsgaard et al., 2017).

Accordingly, this study has two distinct aims: (i) to explore associations between PFO and the content of key proteins relevant to fatty acid metabolism in adipose tissue and skeletal muscle; and (ii) to assess sex differences in the content of these proteins in males and females when matched, and not matched, for cardiorespiratory fitness, physical activity levels and fat mass index classifications.

METHODS

2.1 Study design

This was a cross-sectional study that involved three main visits to a laboratory at the University of Bath, Bath, UK. The study was performed in accordance with the Declaration of Helsinki and was approved by the Research Ethics Approval Committee for Health at the University of Bath (reference EP 16/17 141) and the South West-Bristol NHS Research Ethics Committee (17/SW/0269) and registered on ClinicalTrials.gov: NCT03029364. All participants provided written informed consent before their involvement in the study.

Participants completed two identical trial days (trial A and trial B) separated by 7–28 days (mean ± SD: 11 ± 6 days). Each trial involved the assessment of anthropometric variables, resting metabolic rate, a resting venous blood sample and a FATMAX (exercise intensity at which peak fat oxidation rate occurs) test. Participants then made a third visit (trial C) 2–7 days (mean ± SD: 7 ± 8 days) after trial B, during which body composition was assessed [via a dual energy X-ray absorptiometry scan] and opt-in adipose tissue and/or skeletal muscle
TABLE 1  Participant demographic and lifestyle characteristics (whole sample, n = 36)

| Characteristic                  | Total sample (n = 36) | Range       | Male(n = 21) | Female(n = 15) |
|--------------------------------|-----------------------|-------------|--------------|---------------|
| Age (years)                    | 40 (11)               | 20–63       | 39 (11)§     | 41 (12)§      |
| Ethnicity (% Caucasian)        | 88 – 90               | 87          |              |               |
| Body stature (cm)              | 174.1 (8.6)           | 157.3–191.2 | 179.6 (5.5)§ | 166.3 (5.6)§  |
| Body mass (kg)                 | 72.4 (10.9)           | 52.3–94.0   | 78.5 (8.6)   | 63.8 (7.3)    |
| BMI (kg m⁻²)                   | 23.8 (2.2)            | 19.9–28.5   | 24.3 (2.1)   | 23.0 (2.3)    |
| Body fat (%)                   | 21.8 (8.2)            | 7.7–38.6    | 16.6 (5.0)   | 29.1 (6.0)    |
| Fat mass (kg)                  | 15.4 (5.2)            | 6.1–28.3    | 13.1 (4.3)   | 18.6 (4.8)    |
| Fat mass index (kg m⁻²)        | –                     | –           | 4.1 (1.4)    | 6.8 (1.8)     |
| Fat-free mass (kg)             | 57.0 (12.4)           | 37.8–79.9   | 64.4 (27.9)§ | 44.4 (23.2)§  |
| Body fat (%)                   | 21.8 (8.2)            | 7.7–38.6    | 16.6 (5.0)   | 29.1 (6.0)    |
| Fat-free mass index (kg m⁻²)   | –                     | –           | 4.1 (1.4)    | 6.8 (1.8)     |
| Waist-to-hip circumference     | –                     | –           | 0.85 (0.14)§ | 0.74 (0.32)§  |
| Physical activity level        | 1.73 (0.22)           | 1.39–2.22   | 1.83 (0.14)*** | 1.60 (0.14)*** |
| VO₂peak (l min⁻¹)              | 3.27§                 | 1.64–4.65   | 3.74 (1.59)§ | 2.08 (1.30)*** |
| VO₂peak [ml (kg body mass)⁻¹ min⁻¹] | 42.5 (9.5)          | 26.1–60.6   | 48.4 (6.4)   | 34.2 (6.4)*** |
| VO₂peak [ml (kg FFMI)⁻¹ min⁻¹] | 54.0 (8.9)            | 34.3–72.0   | 56.2 (7.5)   | 48.2 (7.6)*** |
| Peak power output (W)          | 273§                  | 107–380     | 303 (159)§   | 131 (142)§    |
| HRmax (beats min⁻¹)            | 179 (12)              | 148–204     | 179 (13)     | 180 (10)      |
| PFO (g min⁻¹)                  | 0.31 (0.10)           | 0.10–0.62   | 0.35 (0.10)  | 0.26 (0.09)*  |
| PFO [mg (kg FFMI)⁻¹ min⁻¹]     | 5.6 (1.7)             | 2.2–9.9     | 5.4 (1.4)    | 5.9 (2.0)     |
| FATMAX (%VO₂peak)              | 38 (11)               | 23–65       | 39 (42)§     | 38 (32)§      |

Data are presented as the mean (SD) unless otherwise stated below. No whole sample average data are reported for fat mass index and waist-to-hip circumference, nor fat mass index classifications, owing to different male and female thresholds. Abbreviations: BMI, body mass index; FATMAX, exercise intensity at which peak fat oxidation rate occurs; FFM, fat-free mass; HR, heart rate; PFO, peak fat oxidation; VO₂peak, peak oxygen consumption.

†Derived from trial C.
‡Average of trials A and B using dual energy X-ray absorptiometry body fat percentage from trial C.
§Median (range), Mann–Whitney U test on medians.
*P < .05 and
***P < .001 for male versus female.

biopsies were performed. Although this resulted in a relatively long time frame between trial A and trial C, participants were instructed to maintain habitual lifestyle patterns, and the coefficients of variation for resting metabolic rate, VO₂peak (in litres per minute and in millilitres per kilogram per minute), peak power output and maximal heart rate were all <5% (range 1.5 ± 1.1 to 4.3 ± 3.2%), suggesting that the participants were likely to have maintained habitual lifestyle patterns within a reasonable range. Trials were scheduled to be completed after an overnight fast (12 ± 1 h) and at a similar time of day (between 06.30 and 12.30 h, and ±1 h within participants). Additionally, for 48 h preceding each trial, participants were asked to avoid vigorous physical activity and to replicate their diet and physical activity patterns for 48 h.

2.3 | Anthropometrics

Upon each arrival in the laboratory, body stature (Stadiometer, Holtain, Pembrokeshire, UK) and body mass (BC-543 Monitor; Tanita, Tokyo, Japan) were measured. At trial C, waist and hip circumference measures (SECA 201, Hamburg, Germany) and a whole-body dual energy X-ray absorptiometry scan (Discovery; Hologic, Bedford, UK) were also performed.

2.4 | FATMAX test

Before the FATMAX test, resting metabolic rate was assessed by indirect calorimetry, and a 10 ml venous blood sample was collected. The muscle (n = 28) biopsies (n = 24 had both samples). Participant characteristics for the whole sample who had one or more biopsy procedures are displayed in Table 1. For a detailed outline of exclusion criteria, see Chrzanowski-Smith et al. (2020, 2021).

2.2 | Participants

Thirty-six healthy male and female adults (aged 18–65 years; n = 15 females) from a larger study (n = 99; 49 females [Chrzanowski-Smith et al., 2020, 2021]) opted for adipose tissue (n = 32) and/or skeletal
FAT\textsubscript{MAX} test was an incremental graded cycling test to volitional exhaustion completed on a mechanically braked cycle ergometer (Monark Peak Bike Ergomedic 894E, Varberg, Sweden). The graded test involved an initial seven 4 min stages and, if surpassed, stage durations were then shortened to 2 min from stage eight onwards. The initial power output was \textasciitilde 30 or 40 W, and this was increased by \textasciitilde 25 W (excluding the \textasciitilde 10 W increment between the first and second stage in the \textasciitilde 30 W protocol) over the next five and six stages, respectively, and by \textasciitilde 50 W from stage seven onwards (Chrzanowski-Smith et al., 2020).

One-minute expired gas samples, heart rate and ratings of perceived exertion (RPE) were collected in the final minute of the first seven stages and upon the participant’s signal of 1 min remaining before volitional exhaustion. The graded test was used to determine: (i) PFO (in grams per minute; measured values approach (Achten et al., 2002)); (ii) FAT\textsubscript{MAX} (measured values approach ); (iii) peak power output (in watts; power output of the last completed stage, plus the fraction of time in the final non-completed stage, multiplied by the watt increment of that stage); and (iv) $\dot{V}_O_2$\textsubscript{peak} (in millilitres per kilogram per minute).

### 2.5 Metabolic measurements

Expired gas samples were collected into 100–150 litre Douglas bags (Cranlea and Hans Rudolph, Birmingham, UK) via a mouthpiece connected to a two-way, T-shaped non-rebreathing valve (model 2700; Hans Rudolph, Shawnee, KS, USA) and falconia tubing (Hans Rudolph). Indirect calorimetry (Mini MP 5200; Servomex Group, Crowborough, UK) was performed to determine: oxygen consumption (in litres per minute); carbon dioxide output (in litres per minute); ventilation (in litres per minute); the respiratory exchange ratio (RER); and the rate of fat oxidation (in grams per minute; estimated by stoichiometric equations (Frayn, 1983), assuming urinary nitrogen excretion was negligible).

### 2.6 Habitual lifestyle assessment

Habitual levels of physical activity were assessed using a chest-worn physical activity monitor (Actiheart; Cambridge Neurotechnology, Papworth, UK) that participants wore throughout the 7 days before trial A and throughout the 48 h before trial B to assess pretrial physical activity standardization objectively. A minimum of 4 days were required to determine habitual physical activity levels (excluding $n = 1$ participant, for whom only 3 days were available). Habitual energy and macronutrient intake were assessed using a selfweighed diet diary (Pro Pocket Scale\textsubscript{TOP2KG}; Smart Weigh Scales). Participants kept a written record of their food and fluid intakes for \textasciitilde 4 days in the week preceding trial A (including \textasciitilde 1 day at the weekend). Additionally, the 2 days immediately before trial A were recorded in order that participants could replicate this on the 2 days before trial B. Diet records were analysed using Nutritics software (Nutritics, Dublin, Ireland).

### 2.7 Blood sample and analysis

A 10 ml whole blood sample was obtained from an antecubital vein (BD Vacutainer Safety Lok; BD, USA) and split equally into a 5 ml EDTA-coated tube (K3 EDTA; Sarstedt, Germany) and a 10 ml serum/clotting activator tube (Serum Z/10 ml; Sarstedt) for plasma and serum separation, respectively. Samples for plasma separation were immediately centrifuged (1700g for 15 min at 4°C), whereas serum tubes were left to clot for 20–30 min at room temperature before centrifugation (standardized within participant; Heraeus Biofuge Primo R; Kendro Laboratory Products, UK).

The plasma samples were analysed for concentrations of various metabolites and hormones according to the manufacturers’ instructions [coefficients of variations (CV) reported are from the manufacturer except for plasma insulin]. Total plasma non-esterified fatty acids (NEFA; catalogue no. FA115; intra-assay CV <5% and interassay CV <5%), glucose (catalogue no. GL3815; <5 and <6%, respectively), lactate (catalogue no. LC3980; <4 and <5%, respectively) and triglyceride (catalogue no. TR3823; <4 and <4%, respectively) concentrations were run in singular on a Daytona Rx Series clinical chemistry analyser (Randox Laboratories, Crumlin, UK). Total plasma 17β-oestradiol (Elecsys Estradiol III; <7 and <11%, respectively) and progesterone (Progesterone III; <11 and <23%, respectively) concentrations were run in singular on a Cobas 8000 immunochromistry analyser (Modular analytics Cobas e 602; Roche Diagnostics, Rotkreuz, Switzerland). Total plasma insulin concentrations were analysed by an enzyme-linked immuno-sorbent assay (ELISA) kit in duplicate (catalogue no. 900095; Crysatal Chem, IL, USA), with absorption determined by a microplate reader (SPECTROstar Nano; BMG LABTECH, Ortenberg, Germany) at wavelengths specified by the manufacturer (intra-assay CV <2% and interassay CV <24%).

### 2.8 Adipose tissue and skeletal muscle tissue biopsies

Biopsies were taken from adipose tissue and skeletal muscle in a rested state to determine the content of key proteins involved in sequential regulatory sites of lipid metabolism, such as estrogen signalling (ESR1 (ERα)), plasma membrane lipid transport (FABP\textsubscript{pm}), regulation of lipase activity (PLIN1 and ABHD5 (CGI-58)), intracellular lipid hydrolysis (LIPE (HSL) and PNPLA2 (ATGL)), intracellular lipid metabolism (ACSL1) and mitochondrial membrane lipid transport (CPT1B).

Biopsies were taken on the same day (always adipose tissue before muscle). The adipose tissue sample was collected from the subcutaneous abdominal wall region (~5 cm left lateral from the umbilicus) using the needle aspiration technique. The skeletal muscle biopsy sample was obtained using the suction-assisted Bergström percutaneous needle biopsy technique from the vastus lateralis of the participant’s self-reported dominant leg. Both procedures were performed under local anaesthesia [1% lidocaine (lignocaine); Hameln
Pharmaceuticals, UK]. The skeletal muscle samples were quickly frozen in liquid nitrogen and stored at −80°C until subsequent biochemical analysis, whereas the adipose tissue samples were first washed using 0.9% saline (sodium chloride; B. Braun, UK) to remove blood, blood clots and connective tissue. If sufficient adipose tissue sample was obtained (>300 mg), part of the sample (~150–200 mg) was first partitioned and kept for adipose tissue explant experiments, with the remaining sample (or entire sample if insufficient an amount was obtained) snap-frozen in liquid nitrogen.

## 2.9 Adipose tissue explant secretion experiment

Approximately 50 mg of adipose tissue was weighed and placed into each well of a sterile four-well culture plate (176740, Nunclon Delta Surface; Thermo Fisher Scientific, UK) in triplicate (two wells for \( n = 3 \) participants). To each well, endothelial cell basal medium was added (ECBM; Promocell, Germany) supplemented with 0.1% fatty acid-free bovine serum albumin and 1000 units ml⁻¹ penicillin and 0.1 mg ml⁻¹ streptomycin (Sigma Aldrich, UK), at a concentration of ~1 ml per 100 mg of tissue (Travers et al., 2017). The tissue was minced and incubated for 3 h (MCO-18A1C CO₂ incubator; SANYO) at 37°C, 5% CO₂ and 95 ± 5% relative humidity. The media and adipose tissue explants were then transferred to separate Eppendorf tubes and immediately stored at −80°C for later biochemical analysis.

Glycerol concentrations in the collected ECBM were analysed as a marker of ex vivo basal adipose tissue lipolysis rates. An automated analyser was used (Daytona Rx Series; Randox Laboratories) according to the manufacturer’s instructions using a commercially available immunoassay (catalogue no. GY105; Randox Laboratories). All samples were measured in duplicate (intra- and interassay precision were 7.2 and 7.9%, respectively). The concentration of glycerol was expressed per milligram of adipose tissue (in micromoles per litre per milligram of mg tissue per 3 h).

## 2.10 Western blot

Before western blotting, frozen muscle tissue (~60–100 mg) was freeze-dried and powdered, with visible connective tissue removed. The powdered tissue were then mixed in a 1:1 ratio with ice-cold lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% sodium deoxycholate; 0.1% SDS and 0.1% NP-40] supplemented with protease (Thermo Fisher Scientific, UK) and phosphatase inhibitor cocktails (Roche, Switzerland) before homogenization with a dounce homogenizer followed by a 60 min incubation at 4°C with rotation, and 10 min centrifugation at 20,000g, 4°C. In a similar manner, frozen adipose tissue (~50–150 mg) was mixed in a 2:1 ratio with the above-described ice-cold lysis buffer before the samples were homogenized with a Potter–Elvehjem glass/Teflon motorized homogeniser (Thomas Scientific, NJ, USA), followed by the solubilization and centrifugation protocol outlined above. The protein concentrations of the resultant supernatants from the skeletal muscle and adipose tissue samples were then measured using a bicinchoninic acid assay (Thermo Fisher Scientific, UK).

For western blots, 40 and 30 μg of protein for skeletal muscle and adipose tissue samples, respectively, were loaded and separated by SDS-PAGE using 10% acrylamide gels. The gels were then electro-transferred using a semi-dry transfer onto a nitrocellulose membrane (Gelman Sciences, Portsmouth, UK). For both adipose tissue and skeletal muscle samples, immunoblotting was performed with the following primary antibodies: adipose triglyceride lipase (PNPLA2 (ATGL); ab109251; Abcam), oestrogen receptor alpha (ESR1 (ERα); ab75635; Abcam), long chain acyl-CoA synthase 1 (ACSL1; ab76702; Abcam), hormone-sensitive lipase (LIPE (HSL); 4107; Cell Signaling Technology) and actin (A2066; Sigma-Aldrich). In skeletal muscle, the additional following primary antibodies were also used: carnitine palmitoyltransferase 1B (CPT1B; ab134988; Abcam) and fatty acid binding protein plasma membrane [FABP 3 (FABPpm); glutamic-oxaloacetic transaminase 2 (GOT2); ab180162; Abcam]. In adipose tissue, the additional following primary antibodies were also used: perilipin-1 (PLIN1; 9349; Cell Signaling Technology) and comparative gene identification-58 [ABHD5 (CGI-58); abhydrolase domain contain 5 (abhd5); NB110-41576; Novus Biologicals]. All primary antibodies were diluted in Tris-buffered saline (0.09% NaCl, 100 mM Tris–HCl, pH 7.4) with 0.1% Tween 20 (TBS-T) with 1% bovine serum albumin at ~1:1000 dilution, except ESR1 (ERα) used at ~1:300 dilution. After incubation, with anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies (A16104; Thermo Fisher; 1:4000 dilution), immunoblots were detected with enhanced chemiluminescence (ECL) reagent (GE Healthcare, IL, USA) and imaged using EpiChemi II Darkroom (UVP, Upland, USA). The band densities were quantified using Image Studio Lite software (v.5.2; LI-COR, Lincoln, NE, USA). Duplicate samples were run on each gel (excluding skeletal muscle CPT1B and PNPLA2 (ATGL)), and for all participants the actin samples were first normalized to the actin in this duplicate sample (to account for potential inter-gel differences). Protein bands of interest were then normalized to their respective normalized actin. The proteins selected collectively covered the identified regulatory sites of fatty acid oxidation.

## 2.11 Statistical analysis

The relationship between protein content and PFO, expressed in absolute terms (in grams per minute) and relative to FFM (in milligrams per kilogram of FFM per minute), was assessed by bivariate correlation (either a Pearson correlation coefficient (r) and Fisher z 95% confidence intervals (CI), or Spearman’s r and Fieller et al. (1957) 95% CI for parametric and non-parametric data, respectively). The maximum sample size for correlation analysis with the content of proteins in skeletal muscle and adipose tissue was \( n = 28 \) and \( n = 32 \), respectively. To explore potential sex differences in exercise metabolic data, habitual physical activity levels, blood analytes and the content of proteins in skeletal muscle and adipose tissue, either Student’s unpaired t tests or Mann–Whitney U tests were performed.
TABLE 2  Participant demographic and lifestyle characteristics (adipose tissue matched subgroup, n = 14)

| Characteristic                              | Male (n = 7) | Range       | Female (n = 7) | Range       |
|---------------------------------------------|--------------|-------------|----------------|-------------|
| Age (years)                                 | 38 (7)       | 29–48       | 34 (9)         | 24–47       |
| Ethnicity (% Caucasian)                     | 86           | –           | 86             | –           |
| Body stature (cm)                           | 178.0 (4.6)  | 174.2–182.0 | 167.2 (4.6)    | 161.0–174.0 |
| Body mass (kg)                              | 78.1 (9.0)   | 64.1–91.8   | 66.3 (4.5)     | 59.8–73.7   |
| BMI (kg m\(^{-2}\))                        | 24.6 (2.1)   | 21.1–27.6   | 23.8 (2.6)     | 21.2–29.0   |
| Body fat (%)                                | 16.1§        | 13.8–25.5   | 34.2§          | 21.6–38.6   |
| Fat mass (kg)†                               | 14.2 (4.2)   | 8.8–22.2    | 20.8 (5.2)     | 12.9–28.3   |
| Fat mass index (kg m\(^{-2}\))†§           | 4.5 (1.2)    | 2.9–6.8     | 7.5 (2.2)      | 4.6–10.9    |
| Waist-to-hip circumference†§§               | 1.81§        | 1.53–2.08   | 1.75§          | 1.42–1.81   |
| 𝑉_{O_{peak}} (l min\(^{-1}\))              | 3.5 (0.3)**  | 3.2–3.9     | 2.5 (0.4)**    | 1.9–2.9     |
| 𝑉_{O_{peak}} [ml (kg body mass\(^{-1}\)] min\(^{-1}\)] | 43.8§        | 41.5–58.3   | 36.0§          | 31.1–49.2   |
| 𝑉_{O_{peak}} [ml (kg FFM\(^{-1}\)] min\(^{-1}\)] | 55.9 (6.0)   | 49.1–67.8   | 54.2 (4.5)     | 49.1–62.6   |
| Peak power output (W)                       | 309 (35)**   | 265–375    | 175 (43)**     | 131–249     |
| HR\(_{max}\) (beats min\(^{-1}\))          | 175§         | 172–200     | 183§           | 172–191     |
| PFO (g min\(^{-1}\))                        | 0.29 (0.08)  | 0.17–0.36   | 0.32 (0.09)    | 0.19–0.16   |
| PFO [mg (kg FFM\(^{-1}\)] min\(^{-1}\)]    | 4.5 (1.3)*   | 2.7–6.1     | 7.1 (1.9)*     | 4.5–9.9     |
| FATMAX (% 𝑉_{O_{peak}})                     | 31§          | 23–65       | 36§            | 28–60       |

Data are presented as the mean (SD) unless otherwise stated below. Abbreviations: BMI, body mass index; FATMAX, exercise intensity at which peak fat oxidation rate occurs; FFM, fat-free mass; HR, heart rate; PFO, peak fat oxidation; 𝑉_{O_{peak}}, peak oxygen consumption.

† Derived from trial C.
‡ Average of trials A and B using dual energy X-ray absorptiometry body fat percentage from trial C.
§ Median, Mann–Whitney U test on medians.
* P < .05 and
*** P < .001 male versus female.

for parametric or non-parametric data, respectively. Sex differences in the content of these proteins were explored in the whole sample of males and females (range n = 24–32) and in subgroups of males and females matched on an individual level for cardiorespiratory fitness [±5 ml (kg FFM\(^{-1}\)] min\(^{-1}\)], fat mass index classification and physical activity level categories (excluding n = 2 male participants who slightly surpassed the very active habitual physical activity level threshold and were matched to n = 2 female participants who were classified as habitually active with physical activity levels at the higher end of the habitually active threshold; Tables 2 and 3. This matching provided sample) sizes of n = 6 and n = 7 for the analysis of sex differences in the protein content of skeletal muscle and adipose tissue, respectively. Peak fat oxidation and FATMAX (alongside alternative metabolic characteristics and metabolite and hormone concentrations) were derived as an average of trials A and B. Analysis was performed independent of the menstrual cycle and contraceptive use in female participants given that these do not appear to effect estimates of PFO (Chrzanowski-Smith et al., 2020; Frandsen et al., 2020). Descriptive and statistical analyses were run in Microsoft Excel (2013) and IBM SPSS statistics v.27 for Windows (IBM, NY, USA), and graphs were created on GraphPad Prism v.8 software (GraphPad, La Jolla, CA, USA). Data are presented as the mean ± SD for parametric data or the median and range or mean rank for non-parametric data. Statistical significance was accepted at P ≤ 0.05, and sensitivity analyses were performed as appropriate.

3  RESULTS

3.1  Aim 1: Relationship between the content of key proteins involved in fatty acid metabolism in adipose tissue and skeletal muscle with PFO

Statistically significant positive correlations were apparent between PFO (in grams per minute) and the content of ESR1 (ER\(_{α}\)), PLIN1 and
### TABLE 3  Participant demographic and lifestyle characteristics (skeletal muscle matched subgroup, n = 12)

| Characteristic                               | Male (n = 6) | Range       | Female (n = 6) | Range       |
|----------------------------------------------|--------------|-------------|---------------|-------------|
| Age (years)                                  | 37 (6)       | 29–47       | 32 (7)        | 24–42       |
| Ethnicity (% Caucasian)                      | 83           | –           | 83            | –           |
| Body stature (cm)                            | 177.3 (2.2)  | 174.2–180.7 | 164.9 (6.1)   | 157.3–173.5 |
| Body mass (kg)                               | 75.9 (7.3)   | 64.1–87.0   | 65.1 (6.9)    | 54.3–73.7   |
| BMI (kg m⁻²)                                 | 24.1 (1.8)   | 21.1–26.6   | 24.0 (2.8)    | 21.2–29     |
| Body fat (%)†                                | 18.4 (4.4)   | 13.8–25.5   | 29.3 (6.3)    | 21.6–38.4   |
| Fat mass (kg)‡                               | 13.3         | 8.8–22.2    | 19.2           | 12.9–28.3   |
| Fat mass index (kg m⁻²)‡                     | 4.2          | 2.92–6.79   | 6.4           | 4.6–10.9    |
| Fat deficient (%)                            | 1            | –           | 1             | –           |
| Normal adiposity (%)                         | 4            | –           | 4             | –           |
| Excess adiposity (%)                         | 1            | –           | 1             | –           |
| Fat-free mass (%)                            | 62.6         | 55.3–65.9   | 46.1          | 37.8–51.5   |
| Waist-to-hip circumference†                  | 0.82 (0.05)  | 0.76–0.89   | 0.74 (0.05)   | 0.67–0.81   |
| Physical activity level                      | 1.79         | 1.53–2.03   | 1.68          | 1.42–1.81   |
| $V_{\text{O}_2,\text{peak}}$ (l min⁻¹)      | 2.55         | 1.7–2.9     | 3.55          | 3.2–3.7     |
| $V_{\text{O}_2,\text{peak}}$ (ml kg body mass⁻¹ min⁻¹) | 44.4         | 41.5–58.3   | 37.1          | 31.7–49.2   |
| $V_{\text{O}_2,\text{peak}}$ (ml kg FFM⁻¹ min⁻¹) | 56.8 (6.1)   | 49.1–67.8   | 54.2 (5.6)    | 45.5–62.6   |
| Peak power output (W)                        | 298 (21)***  | 265–323     | 177 (50)***   | 107–249     |
| $HR_{\text{max}}$ (beats min⁻¹)              | 180          | 172–200     | 182           | 172–202     |
| PFO (g min⁻¹)                                | 0.32‡        | 0.17–0.36   | 0.32‡         | 0.26–0.46   |
| PFO (mg kg FFM⁻¹ min⁻¹)                      | 4.7 (1.4)    | 2.7–6.1     | 7.3 (1.7)‡    | 5.1–9.9     |
| FATMAX (% $V_{\text{O}_2,\text{peak}}$)     | 35†          | 24–65       | 39†           | 28–60       |

Data are presented as the mean (SD) unless otherwise stated below. Abbreviations: BMI, body mass index; FATMAX, exercise intensity at which peak fat oxidation rate occurs; FFM, fat-free mass; HR, heart rate; PFO, peak fat oxidation; $V_{\text{O}_2,\text{peak}}$, peak oxygen consumption.

1 Derived from trial C.
2 Average of trials A and B using dual energy X-ray absorptiometry body fat percentage from trial C.
3 Median, Mann–Whitney U test on medians.
4 P < 0.05
5 **P < 0.001 and ***P < 0.001 male versus female.

LIPE (HSL) in adipose tissue, but these were not apparent when PFO was expressed relative to FFM (in milligrams per kilogram of FFM per minute; Table 4). No significant correlations were evident between PFO (expressed in absolute rates or relative to FFM) and the content of ABHD5 (CGI-58), PNPLA2 (ATGL) or ACSL1 or ex vivo basal lipolysis rates in adipose tissue (Table 4; all P-values > 0.05).

Significant positive correlations were found between PFO expressed in both absolute terms and relative to FFM and the content of PNPLA2 (ATGL) and CPT1B in skeletal muscle (Figure 1a–d). The protein content of skeletal muscle FABP 3 (FABPpm) was positively correlated with absolute PFO (Table 4) but not relative PFO. No significant correlations were apparent between PFO and the protein content of ESR1 (ERα) or ACSL1 in skeletal muscle (Table 4; all P-values > 0.05).

### 3.2 Aim 2: Exploration of sex differences in PFO and the content of measured proteins in adipose tissue and skeletal muscle

#### 3.2.1 Sexual dimorphism in participant characteristics

Sex differences in participant characteristics for the whole sample and for matched adipose tissue and muscle subgroups are highlighted in Tables 1–3. No sex differences in the subgroups were detected when PFO was expressed in absolute terms ($P > 0.05$; Figure 2a), but in the whole sample males had a higher absolute PFO compared with females ($P \leq 0.01$). Alternatively, females had a higher PFO when expressed relative to FFM compared with males in both the adipose tissue and
Correlations between peak fat oxidation and the content of key fatty acid metabolism proteins in skeletal muscle and adipose tissue (quantified by western blots)

| Protein          | Peak fat oxidation (g min⁻¹) | Peak fat oxidation [mg (kg FFM)⁻¹ min⁻¹] |
|------------------|------------------------------|----------------------------------------|
| Adipose tissue   |                              |                                        |
| ESR1 (ERα) (n = 28) | 0.46 (0.10–0.72)†            | 0.13 (−0.25 to 0.27)                   |
| PLIN1 (n = 28)   | 0.71 (0.46–0.86)†            | 0.12 (−0.27 to 0.48)                   |
| ABHD5 (CGI-58) (n = 23 and 27) | 0.42 (−0.01 to 0.71)†         | 0.18 (−0.22 to 0.52)†                |
| LIPE (HSL) (n = 32 and 30) | 0.38 (0.02–0.65)†            | 0.13 (−0.25 to 0.48)                   |
| PNPLA2 (ATGL) (n = 28) | 0.32 (−0.08 to 0.62)†         | 0.33 (−0.06 to 0.63)                   |
| ACSL1 (n = 28)   | 0.09 (−0.30 to 0.46)         | 0.04 (−0.34 to 0.41)†                 |
| E|F|vivo basal lipolysis rate (n = 24) | −0.38 (−0.69 to 0.04)                  | 0.04 (−0.38 to 0.45)                   |
| Skeletal muscle  |                              |                                        |
| ESR1 (ERα) (n = 19 and 20) | 0.05 (−0.42 to 0.51)         | 0.01 (−0.44 to 0.46)                   |
| FABP 3 (FABPpm) (n = 25 and 26) | 0.44 (0.04–0.72)†            | 0.25 (−0.16 to 0.59)                   |
| ACSL1 (n = 27)   | 0.15 (−0.26 to 0.51)         | 0.23 (−0.17 to 0.57)                   |
| PNPLA2 (ATGL) (n = 27 and 28) | 0.54 (0.19–0.77)†            | 0.41 (0.03–0.68)†                     |
| CPT1B (n = 27 and 28) | 0.52 (0.17–0.75)†            | 0.45 (0.09–0.71)†                     |

Results are given as the Spearman correlation (95% confidence interval) unless stated otherwise. Ex vivo adipose tissue basal lipolysis rates were calculated from the adipose tissue explant secretion experiment that involved the measurement of glycerol concentrations in endothelial cell basal medium after adipose tissue explants were incubated for 3 h (in micromoles per litre per milligram of tissue per 3 h).

Abbreviations: ACSL1, long chain acyl-CoA synthase 1; PNPLA2 (ATGL), adipose triglyceride lipase; ESR1 (ERα), oestrogen receptor alpha; ABHD5 (CGI-58), comparative gene identification-58; FABP 3 (FABPpm), fatty acid binding protein plasma membrane; LIPE (HSL), hormone-sensitive lipase; PLIN1, perilipin 1.

**Pearson correlation coefficient.

*P < 0.05 and **P < 0.01.

skeletal muscle matched subgroups, but a similar rate in the whole sample group (P = 0.014, 0.016 and 0.350, respectively; Figure 2b). The higher PFO rate in females when expressed relative to FFM persisted when normalized for leg FFM (Figure 2c).

3.2.2 | Sexual dimorphism in the content of proteins involved in fat metabolism in adipose tissue and skeletal muscle

No sex differences (all P-values > 0.05) were reported in the total levels of any of the proteins measured in adipose tissue or skeletal muscle in the whole sample (Figures 3 and 4, respectively) or in the respective adipose tissue (Figure 5) and skeletal muscle (Figure 6) matched subgroups. Ex vivo basal adipose tissue lipolysis rates, however, were greater in females both in the whole sample and in the adipose tissue matched subgroup (P < 0.001 and 0.022; Figures 3g and 5g, respectively).

4 | DISCUSSION

In this study, we investigated associations between the abundance of proteins within adipose tissue and skeletal muscle with PFO rates during exercise and explored sexual dimorphism in the content of these proteins. We found that the content of proteins involved in processes relating to intramyocellular triglyceride hydrolysis (PNPLA2 (ATGL)) and mitochondrial fatty acid transport (CPT1B) exhibited moderate and consistent positive correlations with PFO rate expressed in absolute terms and relative to FFM. In contrast, the protein contents of ESR1 (ERα), PLIN1 and LIPE (HSL) in adipose tissue exhibited significant correlations with PFO only when expressed in absolute rates. This suggests that a greater capacity to oxidize fatty acids during exercise has a stronger association with the abundance of proteins in skeletal muscle rather than adipose tissue. Additionally, we showed that although females had a greater rate of PFO (relative to FFM) and basal ex vivo adipose tissue lipolysis rates compared with well-matched males, sexual dimorphism was not evident in the total content of any of the measured proteins in skeletal muscle or adipose tissue. Collectively, these pilot findings help to provide support and insight into the proteins that are likely to be involved in the capacity of an individual to oxidize fat during exercise.

4.1 | Peak fat oxidation and the content of skeletal muscle and adipose tissue proteins

Intramuscular factors, in particular aspects revolving around mitochondria, are suggested to be the major rate-limiting site for fat oxidation during moderate- to high-intensity exercise (Jeppesen...
The prevalent mechanism is believed to be via reduced free carnitine availability during intensive exercise, which inhibits the carnitine-palmitate transferase 1 (CPT1) reaction and the rate of entry of long-chain fatty acids into mitochondria (Lundsgaard et al., 2018; Stephens, 2018; Wall et al., 2011). Consistent with this, we found a moderate correlation between skeletal muscle CPT1B content and PFO rates. Other mitochondrial factors that have previously been associated with PFO, although possibly moderated by training status and cardiorespiratory fitness, are as follows: (i) mitochondrial volume density and content (Dandanell et al., 2018); (ii) markers of mitochondrial capacity (oxidative phosphorylation complexes II–V) (Shaw et al., 2020; Stisen et al., 2006); and (iii) the activity and content of key enzymes in $\beta$-oxidation [$\beta$-hydroxyacyl CoA dehydrogenase ($\beta$HAD)] (Nordby et al., 2015; Rosenkilde et al., 2015; Shaw et al., 2020). Overall, this suggests that the ability to transport fatty acids into the mitochondria alongside the oxidative capacity of skeletal muscle are likely to be important factors that contribute to the capacity of an individual to oxidize fat.

The regulation of PFO is also attributable, in part, to factors involved in the mobilization of IMTG stores. The moderate relationship found between PFO and the content of PNPLA2 (ATGL) in skeletal muscle aligns neatly with a recent study that found positive correlations between PFO and the total abundance of skeletal muscle LIPE (HSL), PNPLA2 (ATGL) and perilipin 5 in trained and untrained males (Shaw et al., 2020). These findings are consistent with the suggestion that during low- to moderate-intensity exercise, when PFO typically occurs, IMTG stores are an important fuel source for energy production (Romijn et al., 1993; van Loon et al., 2003; Watt & Cheng, 2017). Interestingly, however, no associations have been reported between PFO and the content of IMTG or glycogen in trained and untrained subjects (Dandanell et al., 2018; Haufe et al., 2010; Shaw et al., 2020; Stisen et al., 2006), albeit participants were assessed either after a 4 h fast (Shaw et al., 2020) or in a fed state (Haufe et al., 2010; Stisen et al., 2006). Thus, it could be that factors involved in the mobilization of IMTG, rather than the content per se, contribute to the regulation of PFO.

A number of other factors identified in the molecular regulation of skeletal muscle fatty acid oxidation during exercise were also assessed in this study; these included: (i) proteins, enzymes and receptors involved in the mobilization of fatty acids from adipose tissue (i.e., PLIN1, ABHD5 (CGI-58), PNPLA2 (ATGL), LIPE (HSL) and ESR1 (ER$\alpha$)); (ii) sarcolemmal and cytosolic transport of skeletal muscle fatty acids (by FABP 3 (FABPpm) and ACSL1, respectively); and (iii) the content of ESR1 (ER$\alpha$) in skeletal muscle (a mediating factor proposed to upregulate fatty acid oxidation in skeletal muscle). However, despite this physiological rationale, no consistent correlations between these molecular factors and rates of PFO were found here (Table 4). Given that this pilot study is the first to explore many of the above aspects...
FIGURE 2 Peak fat oxidation rates expressed as absolute (a) and relative to total body fat-free mass (b) in females and males in the whole sample and in adipose and muscle subgroups matched for cardiorespiratory fitness, physical activity levels and fat mass index classifications. n = 36 (15 females), n = 14 (seven females) and n = 12 (six females) for the whole sample, matched adipose tissue subgroup and matched muscle subgroup, respectively. (c) Peak fat oxidation rates normalized for leg fat-free mass are also shown from the larger cohort associated with this study (Chrzanowski-Smith et al., 2021). Data are reported as the median and interquartile range for absolute peak fat oxidation rates owing to non-normality evident in the skeletal muscle subgroup, whereas the mean ± SD is reported for relative peak fat oxidation rates alongside the content of certain proteins proving difficult to quantify (namely PLIN1, ABHD5 (CGI-58) and ERα), we suggest that further studies that combine alternative techniques to provide an insight into the localization of these proteins (e.g., through immunohistochemistry) should be conducted. Moreover, factors not explored in the present study, such as capillary density and muscle fibre type composition, have also been shown to be correlated with PFO in young men (Dandanell et al., 2018; Shaw et al., 2020). The influence that skeletal muscle fibre type composition has on PFO could be of particular importance, given that the majority of skeletal muscle proteins assessed in the present study are more highly expressed in type I versus type II muscle fibres. Taken collectively, the research to date suggests that the regulation of PFO is likely to be multifactorial, whereby the upregulation of several mechanisms is likely to be responsible for the increased capacity of an individual to oxidize fat during exercise.

4.2 Sexual dimorphism in fat utilization during exercise

The present study provides new insights alongside confirmatory work on whether sexual dimorphism exists in the total content of key proteins involved in fatty acid metabolism. In sex-comparison studies it is often recommended to match males and females for training status (e.g., maximum oxygen uptake expressed relative to FFM) (Lundsgaard et al., 2017; Skelly & Gibala, 2019; Tarnopolsky, 2008). As such, we performed sex-comparison analyses on the whole sample and in subgroups of males and females matched for cardiorespiratory fitness, habitual physical activity levels and fat mass index classifications. Additionally, given the sexual dimorphism in body composition, the expression of PFO relative to FFM is proposed to be most appropriate when comparing males and females (Amaro-Gahete et al., 2018; Maunder et al., 2018). Aligned with the general consensus, female participants in this study had a higher PFO when expressed relative to FFM compared with well-matched males in both subgroup analyses (Amaro-Gahete, Sanchez-Delgado & Ruiz, 2018; Maunder et al., 2018). Given that the increased FFM in males can often be driven by a higher upper body FFM, this could result in inappropriate normalization of fat oxidation rates to total body FFM during cycling exercise, where the lower limbs are the primary contributors to fat oxidation. However, even when normalizing for leg FFM, females displayed a higher PFO rate than males. This provides confirmation of sex differences in fat oxidation, whereby females display a greater capacity for fat oxidation in a fasted state.

At the level of adipose tissue, ex vivo basal lipolysis rates were higher in females compared with males. This might contribute to the greater PFO reported here in females and corresponds to whole-body data in basal rested conditions (Mittendorfer et al., 2001), albeit not to all ex vivo findings (Lundgren et al., 2008). Nevertheless, this suggests that there might be inherent sexual dimorphism within adipose tissue, independent from important in vivo regulators of adipose tissue metabolism, such as humoral factors and/or blood flow (Lafontan & Langin, 2009; Thompson et al., 2012). Moreover, females generally have greater whole-body lipolysis rates during exercise compared with males (indicated by circulating glycerol concentrations) (Carter et al., 2001; Henderson et al., 2007; Mittendorfer et al., 2002), although the source of increased systemic glycerol concentrations in women (i.e., adipose tissue and/or IMTG lipolysis) remains unclear (Devries, 2016). Furthermore, the present study suggests that sex differences
Sex comparisons, from the whole sample, of the protein content of oestrogen receptor alpha (Erα; a); perilipin-1 (PLIN1; b); comparative gene identification-58 (ABHD5 (CGI-58); c); adipose triglyceride lipase (PNPLA2 (ATGL); d); hormone-sensitive lipase (LIPE (HSL); e); long chain acyl-CoA synthase 1 (ACSL1; f); ex vivo basal lipolysis rates (g); and representative immunoblot for each protein of interest (h). Symbols with the same colour represent the same participant, in order to highlight participants who displayed the highest values for any given protein and their respective values across other proteins. All data are presented as the median and interquartile range (Mann–Whitney U test on medians), except for ABHD5 (CGI-58) and ex vivo basal lipolysis rates, which are shown as the mean ± SD (Student’s unpaired t test).

FIGURE 3

in PFO and fuel metabolism appear to be independent of some important factors involved in the regulation of fatty acid mobilization from adipose tissue (i.e., PLIN1, ABHD5 (CGI-58), LIPE (HSL) and PNPLA2 (ATGL)). Given that this study is, to our knowledge, the first to explore sexual dimorphism in the content of lipolytic gatekeeper proteins (PLIN1 and ABHD5 (CGI-58)) and lipases (PNPLA2 (ATGL) and LIPE (HSL)) in adipose tissue, future studies should provide clarification alongside exploration of alternative factors, such as adipose tissue blood flow and the regulation of different adipose tissue depots.

There are several considerations that should be reflected upon when interpreting the findings from the present study. Firstly, care should be applied when extending these findings to the precise molecular regulation of the capacity of an individual to oxidize fat during exercise, given that the total contents of several proteins were measured in basal, rested conditions and a number of days after the measurement of fat oxidation. Exercise evokes a large physiological stimulus at both the whole-body and tissue levels (Hawley et al., 2014), where the importance of molecular mechanisms in regulating PFO might differ, and the time gap between tissue sampling and measurement of fat oxidation could mean that some associations were missed. However, in a subsidiary analysis of individuals with the smallest time gap between biopsies and measurement of fat oxidation, no new correlations were revealed.

Secondly, the cross-sectional design prevents causality from being inferred, that is, that a greater content of skeletal muscle CPT1B or PNPLA2 (ATGL) leads to a higher PFO. Furthermore, the heterogeneity of the subject population recruited might have impacted the associations reported, particularly given that training status might...
moderate the molecular regulation of fatty acid oxidation (Dandanell et al., 2018; Kiens et al., 1993; Shaw et al., 2020). The quantification of ABHD5 (CGI-58) and PLIN1 was surprisingly difficult given their abundant expression in adipose tissue, where western blot analyses on 3T3-L1 adipocytes confirmed the identification of these bands for quantification.

Finally, the relatively small sample size (especially in some of the subgroup analyses) has the potential to lead to an increased false-negative rate, and the study might be underpowered for some outcomes. Nevertheless, the sex differences in means/medians were small, and thus even if these were underpowered to detect differences statistically, the magnitude of these differences is likely to be small.

To overcome the limitations of the present study, in future studies it might be preferable to take skeletal muscle and adipose tissue biopsies immediately after participants have been exercising at their FATMAX intensity, in order to assess the activation status of proteins involved in lipid metabolism and of mitochondrial enzymes. In addition, the use of stable isotope methods would allow for greater tissue-specific insight into lipid metabolism in vivo by enabling characterization of adipose tissue lipolysis rates and the contribution of fat oxidation from plasma and muscle-based sources.

### 4.3 Conclusion

In the present study, we assessed associations between PFO and the resting content of key proteins involved in fatty acid metabolism in adipose tissue and skeletal muscle. The data demonstrate that factors involved in regulation of IMTG lipolysis (PNPLA2 (ATGL)) and fatty acid entry into the mitochondria (CPT1) were consistently associated with PFO, whereas no such relationships were observed for proteins measured in adipose tissue. This suggests that skeletal muscle has a more important role than adipose tissue in the capacity of an individual to oxidize fat during exercise. Although females consistently displayed increased basal adipose tissue lipolysis rates, no sex differences were found in the content of any of the measured proteins involved in lipid metabolism.
FIGURE 5  Sex comparisons of the protein content of oestrogen receptor alpha (Erα; a); perilipin-1 (PLIN1; b); comparative gene identification-58 (ABHD5 (CGI-58); c); adipose triglyceride lipase (PNPLA2 (ATGL); d); hormone-sensitive lipase (LIPE (HSL); e); long chain acyl-CoA synthase 1 (ACSL1; f); and ex vivo basal lipolysis rates (g), in the subgroup matched for cardiorespiratory fitness, physical activity levels and fat mass index classifications. Symbols with the same colour represent the same participant, in order to highlight participants who displayed the highest values for any given protein and their respective values across other proteins. All data are presented as the median and interquartile range (Mann–Whitney U test on medians), except for ABHD5 (CGI-58), which are the mean ± SD (Student’s unpaired t test). n = 7 females and n = 7 males, except for PLIN1 (n = 7 females, n = 5 males), ABHD5 (CGI-58) (n = 5 females, n = 5 males) and ex vivo basal lipolysis rates (n = 7 females, n = 4 males).

metabolism within adipose tissue or skeletal muscle. This suggests that higher relative rates of fat oxidation during exercise in females than in males are unlikely to be attributable to a higher abundance of key proteins within adipose tissue or skeletal muscle determined in the resting state.

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COMPETING INTERESTS
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AUTHOR CONTRIBUTIONS
O.J.C.-S. and J.T.G. formulated the idea. O.J.C.S. predominately designed the research methodology, with input from R.M.E. and J.T.G. O.J.C.-S. met and recruited all participants, was present on all trial days and collected all experimental data. M.P.T. and R.M.E. helped to collect data from the exercise tests. J.P.W., J.A.B. and J.T.G. performed the tissue biopsy procedures with assistance from O.J.C.-S. O.J.C.-S., E.S. and R.M.E. prepared the tissue samples for analysis. O.J.C.-S. and R.M.E. conducted biochemical analysis on tissue samples with assistance from F.K. O.J.C.S. conducted the statistical analysis with assistance from S.W. O.J.C.-S. wrote the manuscript. All authors interpreted, revised and approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as
FIGURE 6  Sex comparisons of the skeletal muscle protein content of oestrogen receptor alpha (ERα; a); fatty acid binding protein plasma membrane (FABP3; FABPpm; b); adipose triglyceride lipase (PNPLA2; ATGL; c); long chain acyl-CoA synthase 1 (ACSL1; d); and carnitine palmitoyltransferase 1B (CPT1B; e) in the subgroup matched for cardiorespiratory fitness, physical activity levels and fat mass index classifications. Symbols with the same colour represent the same participant, in order to highlight participants who displayed the highest values for any given protein and their respective values across other proteins. All data are presented as the median and interquartile range (Mann–Whitney U test on medians), except for FABP3 (FABPpm), which are the mean ± SD (Student’s unpaired t test). n = 6 females and n = 6 males, except for ESR1 (ERα) (n = 3 females, n = 5 males).

authors qualify for authorship, and all those who qualify for authorship are listed.

DATA AVAILABILITY STATEMENT
The data underpinning this work is available from the University of Bath Research Data Archive. Chrzanowski-Smith, O., Edinburgh, R., Gonzalez, J., 2021. Dataset for “Resting skeletal muscle ATGL and CPT1B are associated with peak fat oxidation rates in men and women but do not explain observed sex differences”. Bath: University of Bath Research Data Archive. Available from: https://doi.org/10.15125/BATH-00729.

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